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Pig-to-baboon liver xenoperfusion utilizing GalTKO.hCD46 pigs and glycoprotein Ib blockade

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Abstract

Background—Although transplantation of genetically modified porcine livers into baboons has yielded recipient survival for up to 7 days, survival is limited by profound thrombocytopenia, which becomes manifest almost immediately after revascularization, and by subsequent coagulopathy. Porcine von Willebrand's factor (VWF), a glycoprotein that adheres to activated platelets to initiate thrombus formation, has been shown to constitutively activate human platelets via their glycoprotein Ib (GPIb) receptors. Here, we report our pig-to-primate liver xenoperfusion model and evaluate whether targeting the GPIb-VWF axis prevents platelet sequestration.

Methods—Twelve baboons underwent cross-circulation with the following extracorporeal livers: one allogeneic control with a baboon liver, 4 xenogeneic controls with a GalTKO.hCD46 pig liver, 3 GalTKO.hCD46 pig livers in recipients treated with α GPIb antibody during perfusion, and 4 GalTKO.hCD46 pig livers pre-treated with D-arginine vasopressin (DDAVP) in recipients treated with α GPIb antibody during perfusion.

Results—All perfused livers appeared grossly and macroscopically normal and produced bile. Xenograft liver perfusion experiments treated with α GPIb antibody may show less platelet

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Disclosure

Richard N. Pierson serves on the Scientific Advisory Board of Revivacor. David Ayares is an employee of Revivacor.

Author contributions

JCL contributed to concept/design, data analysis/interpretation, and drafting article. LB performed concept/design, data analysis/interpretation, statistics, and critical revision of article. TZ performed concept/design and approval of article. ER contributed to concept/design and approval of article. XC performed in vitro assays. RM carried out data analysis/interpretation and statistics. IS performed concept/design and approval of article. KB provided concept/design, essential reagents, and approval of article. ES performed in vitro assays and immunohistochemistry. BM provided concept/design and approval of article. MD provided concept/design and approval of article. DA provided concept/design and approval of article. HD provided concept/design, essential reagents, and approval of article. AMA performed concept/design, data analysis/interpretation, statistics, and critical revision of article. RNP provided concept/design, data analysis/interpretation, statistics, and critical revision of article. RNB provided concept/design, data analysis/interpretation, statistics, and critical revision of article.

sequestration during the initial 2 h of perfusion. Portal venous resistance remained constant in all perfusion experiments. Platelet activation studies demonstrated platelet activation in all xenoperfusions, but not in the allogeneic perfusion.

Conclusion—These observations suggest that primate platelet sequestration by porcine liver and the associated thrombocytopenia are multifactorial and perhaps partially mediated by a constitutive interaction between porcine VWF and the primate GPIb receptor. Control of platelet sequestration and consumptive coagulopathy in liver xenotransplantation will likely require a multifaceted approach in our clinically relevant perfusion model.

Keywords

ex vivo perfusion; liver transplantation; xenotransplantation

Introduction

Pre-clinical large animal studies [1] and human clinical applications [2] of xenogeneic liver transplantation have been limited by profound thrombocytopenia and hemorrhagic complications secondary to platelet sequestration and activation within the xenograft. Both uncontrolled platelet sequestration and coagulation cascade activation are hypothesized to result from molecular incompatibilities between species. Injury to endothelial cells following xenotransplantation enables von Willebrand factor (VWF) to bind the glycoprotein Ib (GPIb) receptor on the platelet cell surface, activating the GPIIb/IIIa receptor [3]. These events lead to fatal, self-propagating activation of the coagulation cascade [4,5]. Coagulation cascade activation is exacerbated in the xenotransplantation setting because porcine VWF, in contrast to human VWF, has been shown to constitutively activate human platelets, resulting in uncontrolled platelet aggregation [6]. Furthermore, negative feedback provided by porcine thrombomodulin is inefficient, and activated protein C levels are not maintained effectively [7–9]. It is also unclear whether porcine tissue factor pathway inhibitor is able to limit initiation of the clotting cascade [10,11]. These factors result in dysregulated activation of the coagulation cascade.

Here, we report the development of an ex vivo liver xenoperfusion circuit and have investigated whether disruption of the interaction between VWF and the GPIb receptor improves platelet sequestration and coagulation cascade dysregulation seen in liver xenotransplantation. An ex vivo perfusion circuit provides an ideal platform to study the effects of isolated genetic and pharmacologic interventions designed to alleviate the consumptive coagulopathy associated with liver xenotransplantation as the xenoliver is isolated within a circuit with access to perfused blood immediately prior to and following organ perfusion. Furthermore, the minimal surgical intervention required on the recipient provides an intuitive path to clinical application should favorable results be obtained in future pre-clinical studies.

A number of strategies of ex vivo liver xenoperfusion have been reported in the past. These studies have been in pre-clinical large animal models utilizing both hepatocyte-based devices [12,13] and whole-organ liver perfusion [14], as well as in limited clinical applications using porcine hepatocytes or whole livers [2,15–18]. In our present report, we

utilize a genetically modified GalTKO.hCD46 porcine liver designed to eliminate hyperacute rejection while simultaneously attempting to interfere with the constitutive activation between VWF and the GPIb receptor by administering D-arginine vasopressin (DDAVP) and α GPIb antibody.

Materials and methods

Animals

Piglets (3 to 20 kg, either gender) genetically engineered to express the human membrane cofactor protein (hCD46) but not the α 1,3-galactosyl transferase gene were supplied by Revivicor (Blacksburg, VA, USA). Baboons (*papio anubis*, 12 to 23 kg, either gender) were received from the University of Oklahoma (Oklahoma City, OK, USA). All procedures were approved by the Institutional Animal Care and Use Committee at the University of Maryland School of Medicine and were conducted in compliance with the National Institutes of Health guidelines for the care and use of laboratory animals.

Experimental groups

One allogeneic control experiment was performed utilizing a baboon liver in the perfusion circuit. Four xenogeneic control experiments were performed, perfusing a porcine liver without DDAVP or α GPIb antibody treatment. The two experimental groups included three animals that were treated with α GPIb antibody alone, and four cases in which the donor pig was treated with DDAVP and the recipient baboon received α GPIb antibody. During rolling review of preliminary results, it was apparent that DDAVP did not have a marked effect when utilized with α GPIb and a fourth α GPIb-only perfusion was not performed.

Recipient surgical procedures

After the induction of anesthesia using intramuscular ketamine (10 mg/kg; Ketaset, Fort Dodge Animal Health, Fort Dodge, IA, USA) and xylazine (1 mg/kg; Rompun, Bayer Pharmaceuticals, Shawnee Mission, KS, USA), recipient baboons were kept under inhalation anesthesia (isoflurane 0.5 to 3%) throughout the surgical procedure. Rectal temperature was maintained at 37 °C utilizing a forced air warming device. Monitoring included invasive and non-invasive arterial pressure, heart rate, electrocardiogram, respiration rate, pulse oximetry, body temperature, and expired partial pressure of carbon dioxide. Euthanasia was performed by exsanguination under deep anesthesia consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

Donor hepatectomy

After the induction of anesthesia using intramuscular ketamine (10 mg/kg) and xylazine (1 mg/kg), donor pigs and baboons were kept under inhalation anesthesia (isoflurane 0.5 to 3%) throughout the surgical procedure. In experimental protocols that required DDAVP administration, donor animals were treated on the day prior to liver procurement with a single dose of intravenous DDAVP (3 μ g/kg; Desmopressin; Sicor Pharmaceuticals, Irvine, CA, USA) under general anesthesia; dosing was repeated on the morning of transplant immediately prior to the start of the procurement procedure. Procurement was performed

under aseptic conditions via laparotomy with exposure of the aorta and vena cava. Heparin (1000 IU/kg donor weight; Heparin Sodium Injection; Sagent, Schaumburg, IL, USA) was given intravenously. Using standard clinical techniques, the infrarenal aorta was cannulated, the supraceliac aorta was cross-clamped, the suprahepatic inferior vena cava was vented with scissors, and the aorta and portal vein then flushed in situ with one liter of cold (4 °C) University of Wisconsin preservation solution (ViaSpan; Belzer UW, Fresenius, Austria). The liver was recovered with care to preserve the suprahepatic vena cava at the level of the right atrium, portal vein at its origin, common bile duct, celiac artery with an aortic Carrel patch, and suprarenal inferior vena cava. The liver was stored in perfusion solution on ice until instrumented and reperfused.

Liver perfusion

All recipient baboons were treated with anti-coagulants (heparin [150 IU/kg IV bolus and continuous infusion to maintain an activated clotting time [ACT] of 300 to 400 s]). Animals that received α GPIIb antibody (10 mg/l of blood volume) were treated prior to the initiation of ex vivo liver perfusion and after 30 min of cross-circulation.

Cold ischemic time was typically 2 to 4 h. The liver perfusion circuit was primed with 50 to 100 cc normal saline, and 100 cc of donor baboon blood of recipient ABO type obtained the evening prior to perfusion (approximately 15 h). The perfusion circuit was initiated prior to running baboon blood through the donor liver to stabilize the animal on cross-circulation. Once the animal was stable, the liver was included in-line with the system. This enabled α GPIIb to circulate through both the recipient animal and the donor baboon blood prior to exposure to the ex vivo liver. The suprahepatic cava of the porcine donor liver was cannulated for drainage, and in-flow catheters were placed in the portal venous and hepatic arterial systems. Vascular catheters were connected by plastic tubing to a cardiopulmonary bypass machine. These pumps were used to regulate portal vein and hepatic artery blood flow at physiologic levels. Upon institution of perfusion, the portal vein flow was adjusted to maintain portal venous pressure <25 mmHg. Although normal portal pressure is 5 to 10 mmHg, higher perfusion pressure was employed due to tubing resistance as well as significant resistance through the perfusion catheter in the vessels. Portal vein flow was maintained between 100 and 320 ml/min. In two outliers noted in the results, a higher perfusion pressure was required to maintain flow greater than 100 ml/min. Hepatic vein effluent was returned by gravity to the blood reservoir from which a separate third pump led the blood back to the recipient. The donor liver was placed in a sterile basin with warm sterile saline (intravenous infusion grade) and maintained at 37 °C with a water bath. The Digimed System Integrator (Micro-Med, Louisville, KY, USA) was used to provide a continuous measurement of both hepatic arterial and portal venous pressures. The perfusion apparatus is illustrated in Fig. 1.

Recipient baboon hemodynamics were monitored continuously and used to regulate administration of volume and inotropic agents to support normal preload (central venous pressure >4 mmHg) and peripheral perfusion (mean arterial blood pressure >60 mmHg, capillary refill <2 s). Blood samples were obtained from the arterial line and the donor liver hepatic vein at defined intervals.

The recipient baboon was euthanized if the recipient exhibited severe hypotension (mean blood pressure consistently below 40 mmHg) or hypoxemia (oxygen saturation below 80%) despite high-dose inotropic and optimized ventilatory support or when an arbitrarily defined target duration of support (6 h) was reached.

α GPIIb antibody administration

A murine anti-human GPIIb-blocking antibody (6B4) fragment (Fab, 1 mg/kg, provided by H. Deckmyn, Laboratory for Thrombosis Research, Belgium) [19] was administered prior to instituting liver perfusion, aiming to obtain a target concentration of 10 μ g/ml of blood. As preliminary dosing studies demonstrated that plasma concentration and GPIIb receptor occupancy dropped after 30 min, an additional dose of 1 mg/kg was given 30 min following the initiation of liver perfusion [20,21].

Sampling regimen

Baseline blood samples were taken before and after equilibration of the perfusion circuit with the baboon (prior to the initiation of liver perfusion) and after 5, 15, 30, 60, 120, 180, 240, 300, and 360 min of liver perfusion. The sample collected after the recipient baboon's blood had been circulated through the perfusion circuit with the donor liver was identified as time zero (T 0). An organizational flow chart is depicted in Fig. 2.

Hematologic analysis

Analysis of peripheral blood white blood cell, neutrophil, and platelet counts, as well as plasma liver function test values, was performed in the University of Maryland Medical Center clinical laboratory.

Histology and immunohistochemistry

Pig liver tissue samples were collected in situ in the donor, before xenoperfusion, and after 30, 60, 120, 180, 240, 300, and 360 min of perfusion. Wedge liver biopsies were obtained from the liver periphery in an effort to maximize specimen size while minimizing bleeding from the biopsy site, and specimens were trisected. Formalin-fixed, paraffin-embedded sections were stained with hematoxylin and eosin for light microscopy and analyzed qualitatively for intravascular thrombosis and interstitial hemorrhage. Two additional pieces were snapfrozen in liquid nitrogen, one without further manipulation and the other with optimal cutting temperature (OCT) compound. Samples were stored at -80°C .

Frozen tissue sections from OCT-infused biopsy specimens (30' and final biopsies) were assessed by immunohistochemistry with monoclonal antibodies against CD41 (platelet marker, Immunotech, Marseilles, France) at 1: 100 and with DAPI (Sigma, St. Louis, MO, USA) nuclear counterstaining. The IHC score is a blinded, qualitative scoring system of platelet deposition in which the deposition of platelets in the liver tissue was quantified by the staining intensity (from 0 to 3), taking into account both the extent (proportion of tissue surface with detectable staining) and intensity of staining, relative to standard positive and negative control sections [22].

Beta-thromboglobulin and thrombin–anti-thrombin

Blood for coagulation assays was collected in CTAD vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) after breaking vacuum. Platelet free plasma was collected and stored at -80°C until use. Assays for β -thromboglobulin (Asserachrom β -TG; Diagnostica Stago, Asnieres, France) and thrombin–anti-thrombin (Enzygnost TAT micro; Siemens, Marburg, Germany) were performed according to the manufacturer's instructions. The normal reference range for human β -TG and TAT is <50 IU/ml and <2.0 to 4.2 mg/l, respectively.

Statistical analysis

Unless otherwise mentioned, all data are presented as means and standard error of the mean (SEM) for all variables except for the survival time, which is expressed as median. Continuous variables were checked for normality. Variables that were normally distributed were assessed with a one-way analysis of variance and Student's *t*-test. For values that failed to pass the normality test, the Mann–Whitney Rank Sum Test was used. Repeated measures testing using multiple intervals from 5 to 300 min using raw data and deltas was utilized to analyze platelet counts during perfusion. Each animal had one value at each measured time point. P-values less than 0.05 were considered statistically significant. All tests were two-tailed. All statistical analyses were performed on a personal computer with the statistical package SigmaPlot for Windows (Version 11.0; Systat Software, Inc., San Jose, CA, USA).

Results

Graft and recipient survival

Perfused grafts demonstrated normal gross appearance and produced bile during ex vivo perfusion in all cases. Eight of 12 perfusion experiments reached the 6-h experimental end point. In four cases, recipient baboons developed hemodynamic instability with increasing oxygen and pressor requirements. Two of these cases were xenogeneic control liver perfusion experiments without anti-platelet therapy, one had been treated with αGPIIb and one had been treated with DDAVP and αGPIIb . The hemodynamic instability seemed to occur secondary to generalized coagulopathy. As banked blood was unavailable during the perfusion, the instability of the animal warranted termination of the experiment. No case demonstrated evidence of significant organ ischemia, infarction, or necrosis during perfusion.

Thrombocytopenia

As shown in Fig. 3A, platelet counts fell in all animals within 5 min of perfusion, and this decline was maintained throughout the perfusion period. While the allogeneic control experiment showed a platelet decline to roughly 60% of its original value by the conclusion of the experiment, the thrombocytopenia was most marked in the xenoperfusions. Treatment groups (both αGPIIb antibody and αGPIIb /DDAVP combination treatment) tended to maintain higher platelet counts during the first 2 h of perfusion as compared to the xenogeneic controls, but platelet counts subsequently fell to comparable levels to the xenogeneic control. This difference did not reach statistical significance ($P = 0.105$). The

mean platelet count at all time points was lowest in the xenogeneic control group. Bleeding at incision and line insertion sites was not observed in the perfused baboons and did not contribute to the end point in any experiment.

The addition of pre-perfusion donor DDAVP therapy to α GPIb antibody therapy did not appear to alter post-perfusion platelet counts relative to α GPIb alone.

Platelet and coagulation cascade activation

The platelet activation (β TG, Fig. 3B) and thrombin generation (TAT, Fig. 3C) markers showed compatible results in regards to platelet activation in allo- and xenoperfusion experiments. In the alloperfusion control experiment, β TG and TAT levels remained within physiologic range, consistent with minimal platelet activation, and showed little increase over time. On the other hand, β TG and TAT became progressively elevated in all xenoperfusions.

Portal vascular resistance

Perfusion pressure was maintained within physiologic range, and flow rates monitored via the perfusion system. Portal vascular resistance was calculated by dividing the infusion pressure by the flow rate. Vascular resistance remained remarkably stable throughout the duration of perfusion in nearly all cases (Fig. 4). In one untreated xenogeneic control animal in which the experiment was terminated early due to hemodynamic instability, the portal vein resistance increased at a pace consistent with the animal's increasing instability.

Two livers obtained from the smallest donors demonstrated notably increased resistance throughout the perfusion. This resistance remained stable throughout the perfusion.

Leukocyte sequestration

Polymorphonuclear leukocyte (PMN) levels were noted to decrease in all animals upon initiation of the perfusion system. As shown in Fig. 5, PMN levels began to increase in all animals within 60 min of liver perfusion. The degree of recovery was variable, with the allogeneic control levels doubling relative to the pre-perfusion levels and the α GPIb-treated animals reaching levels of 50% of baseline.

Liver function tests

While alanine transaminase levels in the hepatic vein effluent of the perfused liver trended upward throughout the perfusion, the levels remained within the normal range for a baboon (Fig. 6A). On the other hand, in all xenoperfusions, the aspartate transaminase levels markedly increased throughout the experiment (Fig. 6B). By the conclusion of perfusion, all groups demonstrated aspartate aminotransferase (AST) levels between 400 and 800 IU/l. In the single allogeneic control, unexplained significant ischemia/reperfusion injury was noted, with alanine aminotransferase (ALT) levels reaching 190 IU/l and AST levels reaching 574 IU/l.

Xenograft histopathology

Biopsies were obtained at the specified time points in all animals and all specimens examined. Representative H&E staining of the donor liver is shown in Fig. 7. The allocontrol liver demonstrated intact parenchyma with no significant changes during perfusion (Fig. 7A–D). The untreated xenocontrol liver (Fig. 7E–H) demonstrated progressive parenchymal congestion and polymorphonuclear cell infiltrate as the perfusion proceeded. Ultimately, necrosis was noted after 240 min of perfusion (Fig. 7G,H). In both α GPIb antibody and α GPIb/DDAVP treatment groups, the xenoperfused livers demonstrated polymorphonuclear infiltrate at all time points (Fig. 7I–P, respectively). Despite this infiltrate, necrosis was not seen in either treatment group, and overall parenchymal congestion was decreased as compared to the xenocontrol group.

Representative recipient necropsy specimens are shown from the native liver, lung, and kidney from animals undergoing no treatment (Fig. 8A–C), α GPIb antibody treatment (Fig. 8D–F), and combined α GPIb/DDAVP treatment (Fig. 8G–I). No thrombi or platelet aggregates were identified in recipient tissue.

Immunohistochemistry

CD41 staining demonstrated that platelet deposition occurred in the donor liver within 30 min of perfusion. Staining increased in intensity and amount over the duration of the perfusion, suggesting ongoing platelet deposition in allogeneic and xenogeneic control perfusions as well as in both treatment groups (Fig. 9A,B). While there was no statistically significant difference in IHC scoring between control and experimental treatment groups (Fig. 9C) at 30 or 360 min, platelet deposition tended to be lower in the allocontrol perfusion at 30 min.

Discussion

The concept of xenogeneic liver perfusion serving as a bridge to transplantation is clearly not novel. Prior to the establishment of modern surgical approaches to liver transplantation, it was thought that this method could serve as a bridge to recovery in acute liver failure, and, as noted earlier, a host of studies were performed to investigate its feasibility. This approach was revisited 10 years ago in a model of pig-to-baboon liver cross-circulation similar to the model employed in this study [23]. Initial studies involving xenoperfusion of a porcine liver with baboon blood were limited by a severe xenoimmune response leading to hemolysis [18,24,25]. This model was refined by the incorporation of the transgenic hDAF porcine strain. The perfused liver produced bile, had normal gross appearance, and demonstrated mild injury on histology. Nonetheless, the success of this strategy was again limited by hemolysis and severe thrombocytopenia. Here, we have expanded on these earlier works to develop a combined genetic and pharmacologic strategy in an effort to both eliminate problems with the uncontrolled xenogeneic immune response (GalTKO.hCD46) and to attenuate consumptive coagulopathy associated with liver xenotransplantation (DDAVP, α GPIb).

While there has thus far been a limited world experience with liver transplantation utilizing Gal- TKO donor pigs, the common roadblock to success in this model has been the dysregulation of the coagulation system. The most recent investigations by the University of Pittsburgh revealed profound thrombocytopenia and death of recipients within 4 to 7 days [1]. Nonetheless, this study demonstrated the synthetic function of the xenograft by detecting the production of pig-specific proteins such as fibrinogen, plasminogen, albumin, and haptoglobin on Western blot. Factor II, V, VII, VIII, IX, X, XI, and XII levels, as well as anti-thrombin levels, were maintained following transplant [26]. Short half-lives of coagulation factors provided evidence for ongoing production following transplantation. These are key findings in support of the ability of the liver xenograft to provide life-supporting biochemical and physiologic function once the unopposed activation of the clotting cascade and platelet sequestration has been overcome.

Our data suggest that the thrombocytopenia associated with liver xenotransplantation is multifactorial and that a combination of pharmacologic therapies and genetic modifications to the porcine donor liver will be required to ameliorate it. While the administration of α GPIb antibody did not result in a statistically significant improvement in thrombocytopenia, there was a non-significant trend toward a transient maintenance of higher platelet counts during the first 2 h of perfusion.

While the lack of a profound effect is disappointing, this is not surprising given the complexity and redundancy of the coagulation cascade. The potential for future therapeutic application of our model was validated in this study, our first utilizing ex vivo liver perfusion. Treated animals initially tolerated xenoperfusion without significant systemic effects, and without early hemodynamic instability limiting the experiment. Xenografts also demonstrated normal gross and histologic appearance and produced bile.

Despite encouraging results in the development of our model, platelet sequestration and consumptive coagulopathy remain the current barriers to success in liver xenotransplantation in a pig-to-baboon model. These challenges presently would be expected to prevent widespread clinical application of porcine liver xenotransplantation. This report demonstrates that our efforts to target a single component of the platelet activation cascade, interference with the VWF-GPIb pathway with α GPIb antibody, will likely not sufficiently address consumptive coagulopathy.

The most interesting finding of this study may be that the α GPIb antibody treatment seemed to delay platelet sequestration within the first 2 h of xenoperfusion ($P = 0.10$). Despite an additional dose of antibody at 30 min, after 2 h of perfusion, platelet levels declined and approached that seen in xenogeneic control perfusions. This suggests the need for additional dosing with antibody at latter stages of perfusion. Additional experience in this model will be necessary to clarify the effects, if any, of DDAVP administration. The slow, but steady, decline in platelet counts noted in the allogeneic control shows that the perfusion system does contribute to platelet sequestration at some level. Nonetheless, this effect is much less marked than that seen in the xenoperfusions. Immunohistochemical staining for CD41 demonstrated similar IHC scoring following 360 min of perfusion in all xenogeneic groups. This finding suggests that platelets that are lost during the early phase of perfusion are

deposited and sequestered in the porcine liver, and that this sequestration continues throughout the perfusion. Although there appears to be a transient decrease in platelet sequestration in the first 2 h of perfusion (as measured by total platelet count), this difference does not manifest itself in terms of platelet deposition within the xenograft. We were unable to detect platelet deposition in other tissues on necropsy via H&E.

As would be expected, animals undergoing xenoperfusion demonstrated higher levels of platelet activation and thrombin generation (as measured by the surrogate markers β TG and TAT), as compared to the allocontrol. This effect was not eliminated when platelet activation and thrombin generation were controlled for differential platelet counts (data not shown). The effect of DDAVP on platelet activation was reported by other groups, and platelet activation in a porcine pulmonary ex vivo xenoperfusion model was attenuated by DDAVP administration [27]. This effect was not statistically significant in our model. We did not obtain liver biopsies in the earliest experiments due to concern for potential liver hemorrhage upon initiation of the perfusion circuit. This limited our ability to compare tissue levels of VWF via immunohistochemistry. Utilizing our lung xenoperfusion tissues as a proxy, we found no obvious difference in VWF staining by immunohistochemical analysis (not shown). The dose or timing of DDAVP treatment we used may be suboptimal, and further optimization may be needed. Although no beneficial effect of DDAVP administration was detectable, it did not appear to lead to clinical bleeding complications. Although any potential effect may be too small to detect, the cost to potential benefit ratio is in favor of its continued use, and we are planning to use it in upcoming xenoperfusion experiments. We intentionally did not include a DDAVP-alone treatment group due to the tremendous cost of each perfusion and ethical concerns regarding the use of primates in a treatment arm with little potential effect. The lack of platelet activation noted in the allogeneic controls, an expected finding, lends credence to the validity of the ex vivo perfusion model and the associated assays.

We were encouraged by the perfusion characteristics of the porcine liver in the system. Our study did not demonstrate a significant increase in portal vein resistance during perfusion, as has been noted in isolated lung xenoperfusion experiments performed by members of our group (data not shown). We would expect that severe, ongoing liver insults from ischemia–reperfusion injury and the xenoimmune response would lead to edema within the graft and increased resistance over time. However, the resistance over time was remarkably stable (and the lack of massive hepatocyte injury was confirmed by relatively modest increases in transaminases over time). It is our feeling that the initial resistance encountered is most related to the size of the perfusion catheter used in the experiment. One major limitation of our technique is that flow is measured between the infusion roller pump and the infusion catheter, and not beyond the catheter. As we gained experience and refined our technique, we tended toward larger catheters to minimize this effect. It is for this reason that we feel that the most critical value is actually the change in resistance over time. As the model artificially creates resistance to flow, we would not expect any relationship with normal values (typically 5 to 10 mmHg). The high resistance noted in the two smallest donor livers is likely due to the smaller perfusion catheters necessitated by the anatomic constraints of the smaller portal vein in such donor livers.

The recovery of PMN cell counts following an initial sequestration on initiation of the perfusion circuit has not been noted with isolated organ perfusion in a lung model. The initial decline is likely secondary to both a dilutional effect of the circuit (blood and saline primed) as well as sequestration within the graft, which may be linked to platelet activation. The recovery of PMN counts during the perfusion likely reflects a stress response from the recipient animal prompted both by the surgical procedure and cross-circulation.

Our findings of a disproportionate rise in the AST relative to the ALT in xenoperfusion experiments confirm results noted in other experiments [28]. The mechanisms underlying this phenomenon have yet to be elucidated. It is not unexpected that there is a degree of ongoing injury in response to xenoantigens and to ischemia–reperfusion injury being manifested by a steadily increasing transaminitis.

It should not be expected that any one intervention will completely eliminate the consumptive coagulopathy associated with liver xenotransplantation. Although it may be transiently attenuated, the profound, immediate sequestration of platelets following exposure of baboon blood to the porcine liver endothelium has not been eliminated by α GPIb antibody treatment. Additional therapies will be required, and studies evaluating pig livers that additionally express human thrombomodulin, endothelial protein C receptor, CD39, and/or human tissue factor pathway inhibitor are presently planned. The generation of a porcine donor that expresses primate VWF has the potential to eliminate primate GPIb–porcine VWF interaction, while leaving intact the primate GPIb response to primate VWF.

Rapid advancements in the generation of Gal-TKO donor pigs with additional genetic modifications provide exciting possibilities for future experiments. Studies utilizing these novel pigs are presently underway using a life-supporting ex vivo perfusion model in which the recipient baboon has undergone total hepatectomy with portacaval shunting. In addition to the parameters noted in this report, we are monitoring the recipient for evidence of porcine protein production, DDAVP expression, and bile volume and characteristics.

While a number of such hurdles have yet to be overcome, we speculate that a combination of pharmacologic therapies and genetic modifications will prove necessary and sufficient to permit clinical assessment of the therapeutic potential of liver xenografts.

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Abbreviations

ALT	alanine aminotransferase
AST	aspartate aminotransferase
βTG	β -thromboglobulin

DDAVP	D-arginine vasopressin
ELISA	enzyme-linked immunosorbent assay
Fab	fragment, antigen- binding
GalTKO	α 1,3-galactosyl transferase knockout
GPIb	glycoprotein Ib
hCD46	human membrane cofactor protein
OCT	optimal cutting temperature
PMN	polymorphonuclear leukocyte
PVR	portal venous resistance
SEM	standard error of the mean
TAT	thrombin–anti-thrombin
VWF	von Willebrand's Factor

References

1. Ekser B, Long C, Echeverri GJ, et al. Impact of thrombocytopenia on survival of baboons with genetically modified pig liver transplants: clinical relevance. *Am J Transplant*. 2010; 10:273–285. [PubMed: 20041862]
2. Pascher A, Sauer IM, Hammer C, Gerlach JC, Neuhaus P. Extracorporeal liver perfusion as hepatic assist in acute liver failure: a review of world experience. *Xenotransplantation*. 2002; 9:309–324. [PubMed: 12199863]
3. Schulte am Esch J II, Cruz MA, Siegel JB, Anrather J, Robson SC. Activation of human platelets by the membrane- expressed A1 domain of von Willebrand factor. *Blood*. 1997; 90:4425–4437. [PubMed: 9373253]
4. Robson SC, Cooper DK, d'Apice AJ. Disordered regulation of coagulation and platelet activation in xenotransplantation. *Xenotransplantation*. 2000; 7:166–176. [PubMed: 11021661]
5. Cowan PJ, Roussel JC, d'Apice AJ. The vascular and coagulation issues in xenotransplantation. *Curr Opin Organ Transplant*. 2009; 14:161–167. [PubMed: 19469030]
6. Schulte Am Esch J II, Robson SC, Knoefel WT, Hosch SB, Rogiers X. O-linked glycosylation and functional incompatibility of porcine von Willebrand factor for human platelet GPIb receptors. *Xenotransplantation*. 2005; 12:30–37. [PubMed: 15598271]
7. Kopp CW, Grey ST, Siegel JB, et al. Expression of human thrombomodulin cofactor activity in porcine endothelial cells. *Transplantation*. 1998; 66:244–251. [PubMed: 9701273]
8. Siegel JB, Grey ST, Lesnikoski BA, et al. Xenogeneic endothelial cells activate human prothrombin. *Transplantation*. 1997; 64:888–896. [PubMed: 9326416]
9. Roussel JC, Moran CJ, Salvaris EJ, et al. Pig thrombomodulin binds human thrombin but is a poor cofactor for activation of human protein C and TAFI. *Am J Transplant*. 2008; 8:1101–1112. [PubMed: 18444940]
10. Kopp CW, Siegel JB, Hancock WW, et al. Effect of porcine endothelial tissue factor pathway inhibitor on human coagulation factors. *Transplantation*. 1997; 63:749–758. [PubMed: 9075849]
11. Lee KF, Salvaris EJ, Roussel JC, et al. Recombinant pig TFPI efficiently regulates human tissue factor pathways. *Xenotransplantation*. 2008; 15:191–197. [PubMed: 18611227]
12. Matsushita T, Amiot B, Hardin J, Platt JL, Nyberg SL. Membrane pore size impacts performance of a xenogeneic bioartificial liver. *Transplantation*. 2003; 76:1299–1305. [PubMed: 14627907]

13. Nyberg SL, Yagi T, Matsushita T, et al. Membrane barrier of a porcine hepatocyte bioartificial liver. *Liver Transpl.* 2003; 9:298–305. [PubMed: 12619028]
14. Waldman JP, Vogel T, Burlak C, et al. Blocking porcine sialoadhesin improves extracorporeal porcine liver xenoperfusion with human blood. *Xenotransplantation.* 2013; 20:239–251. [PubMed: 23822217]
15. Sauer IM, Kardassis D, Zeillinger K, et al. Clinical extracorporeal hybrid liver support–phase I study with primary porcine liver cells. *Xenotransplantation.* 2003; 10:460–469. [PubMed: 12950989]
16. Chari RS, Collins BH, Magee JC, et al. Brief report: treatment of hepatic failure with *ex vivo* pig-liver perfusion followed by liver transplantation. *N Engl J Med.* 1994; 331:234–237. [PubMed: 8015570]
17. Horslen SP, Hammel JM, Fristoe LW, et al. Extracorporeal liver perfusion using human and pig livers for acute liver failure. *Transplantation.* 2000; 70:1472–1478. [PubMed: 11118093]
18. Nishitai R, Ikai I, Terajima H, et al. Influence of extracorporeal porcine liver perfusion on nonhuman primates: minimizing hemolysis improves subsequent survival. *Liver Transpl.* 2001; 7:615–622. [PubMed: 11460229]
19. Cauwenberghs N, Meiring M, Vauterin S, et al. Anti-thrombotic effect of platelet glycoprotein Ib-blocking monoclonal antibody Fab fragments in nonhuman primates. *Arterioscler Thromb Vasc Biol.* 2000; 20:1347–1353. [PubMed: 10807753]
20. Wu D, Meiring M, Kotze HF, Deckmyn H, Cauwenberghs N. Inhibition of platelet glycoprotein Ib, glycoprotein IIb/IIIa, or both by monoclonal antibodies prevents arterial thrombosis in baboons. *Arterioscler Thromb Vasc Biol.* 2002; 22:323–328. [PubMed: 11834536]
21. Fontayne A, Meiring M, Lamprecht S, et al. The humanized anti-glycoprotein Ib monoclonal antibody h6B4-Fab is a potent and safe antithrombotic in a high shear arterial thrombosis model in baboons. *Thromb Haemost.* 2008; 100:670–677. [PubMed: 18841291]
22. Nguyen BN, Azimzadeh AM, Zhang T, et al. Life-supporting function of genetically modified swine lungs in baboons. *J Thorac Cardiovasc Surg.* 2007; 133:1354–1363. [PubMed: 17467457]
23. Matsushita T, Ikai I, Nishitai R, et al. Suppressed complement activation in human decay accelerating factor transgenic porcine liver cross-circulated with nonhuman primates. *Transplantation.* 2003; 75:1807–1812. [PubMed: 12811238]
24. Terajima H, Shirakata Y, Yagi T, et al. Successful long-term xenoperfusion of the pig liver: continuous administration of prostaglandin E1 and insulin. *Transplantation.* 1997; 63:507–512. [PubMed: 9047142]
25. Uesugi T, Ikai I, Yagi T, et al. Evaluation of ammonia and lidocaine clearance, and galactose elimination capacity of xenoperfused pig livers using a pharmacokinetic analysis. *Transplantation.* 1999; 68:209–214. [PubMed: 10440389]
26. Ekser B, Echeverri GJ, Hassett AC, et al. Hepatic function after genetically engineered pig liver transplantation in baboons. *Transplantation.* 2010; 90:483–493. [PubMed: 20606605]
27. Kim YT, Lee HJ, Lee SW, et al. Pre-treatment of porcine pulmonary xenograft with desmopressin: a novel strategy to attenuate platelet activation and systemic intravascular coagulation in an *ex vivo* model of swine-to-human pulmonary xenotransplantation. *Xenotransplantation.* 2008; 15:27–35. [PubMed: 18333911]
28. Ekser B, Gridelli B, Cooper DK. Porcine alanine transaminase after liver allo- and xenotransplantation. *Xenotransplantation.* 2012; 19:52–55. [PubMed: 22360753]

Fig. 1.

Diagram of the perfusion system. A cardiopulmonary bypass machine was primed with recipient blood-type compatible allogeneic blood. The femoral vein of the recipient baboon provided inflow to the bypass machine. The porcine liver was then perfused through pressure-controlled portal venous and hepatic arterial lines. The suprahepatic cava was cannulated, and the blood returned to the perfusion circuit. The blood was warmed and returned to the recipient baboon via the internal jugular vein. The common bile duct was cannulated separately to monitor bile production.

Fig. 2.

Experimental timeline. In experimental treatment groups, αGPIb was administered prior to initiation of the extracorporeal circuit. After establishing hemodynamic stability on the perfusion circuit, the xenoliver was perfused in-line. αGPIb was re-dosed after 30 min of xenoperfusion. Blood sampling was performed at the noted intervals.

Fig. 3.

Platelet and coagulation monitoring. (A) The allogeneic control demonstrated maintenance of adequate platelet counts throughout the perfusion, with a slow, expected decline in total counts over the perfusion. While all xenogeneic perfusion experiments showed marked platelet count decline, treated animals (α GPIb+/-D-arginine vasopressin [DDAVP]) may have maintained platelet counts at a higher level over the first 120 min of perfusion before falling toward untreated levels ($P = 0.105$). (B) β -thromboglobulin (β TG) level changes over the perfusion. β TG levels remained low in allogeneic perfusion, demonstrating minimal change in platelet activation. Conversely, all xenogeneic perfusion experiments demonstrated increased levels of platelet activation over baseline. (C) Thrombin-anti-thrombin (TAT) plasma levels mirrored β TG findings. TAT levels remained within the normal human range in the alloperfusion and showed minimal change over time, with minimal thrombin generation, but xenoperfusions demonstrated increased levels of coagulation activation.

Fig. 4.

Portal vein resistance. Portal venous resistance remained stable throughout the perfusion in α GPIb/D-arginine vasopressin (DDAVP)-treated (A) and α GPIb-treated (B) groups, as well as in the untreated xeno- and allogeneic control animals (C). Particularly high portal vein resistance was noted at baseline in pigs #4 and #6. These two donor pigs were markedly smaller than the majority of those used in other perfusions. In these particular cases, the smaller donor livers required smaller perfusion catheters than those used in the other perfusions due to anatomic constraints. Nonetheless, even in these two perfusions, portal vein resistances remained stable over most of the experimental period.

Fig. 5. Polymorphonuclear (PMN) leukocyte counts. Although PMNs tended to be sequestered on initial establishment of perfusion, counts rebounded during the perfusion. (A) shows the first 30 min of perfusion. It is likely that there is an initial dilutional effect from the circuit, followed by sequestration within the graft (noted on immunohistochemistry). The recovery of PMN counts during the latter stages of perfusion (B) may reflect a native stress response from the recipient animal.

Fig. 6.

A steady, gradual rise in alanine aminotransferase (ALT, (A)) and aspartate aminotransferase (AST, (B)) demonstrates a degree of ongoing injury from ischemia/reperfusion. Column 1 shows the changing transaminases over the first 60 min, while column 2 shows the changes over the course of the perfusion. The level of injury is comparable to that seen in whole-organ transplant. The allogeneic control data were censored due to an unexplained disproportional elevation in the ALT.

Fig. 7.

Ex vivo perfused liver biopsy specimens (stained with H&E) are shown at 0, 60, and 240 min at 20× magnification, and at 40× magnification at 240 min following the initiation of perfusion. (A–D) show the untreated allocontrol over time. At all time points, the allograft demonstrated intact hepatic parenchyma with no significant changes. (E–H) show the untreated xenocontrol at the same time points. Progressive parenchymal congestion (stars) and polymorphonuclear cellular infiltrate (arrows) is noted as the length of perfusion increases. Necrosis was noted after 240 min of perfusion. (I–L) show a xenograft which received αGPIIb antibody alone, and (M–P) show a xenograft which was treated with both αGPIIb and D-arginine vasopressin (DDAVP). In these specimens, polymorphonuclear cellular infiltrate is noted to a lesser degree than that seen in the xenograft (arrows), and there is no necrosis and decreased parenchymal congestion in the treated groups.

Fig. 8.

Neither platelet aggregates nor fibrin thrombi were identified in recipient tissues at necropsy. Representative specimens are shown from the native liver, lung, and kidney from animals undergoing no treatment (A–C), αGPIIb antibody treatment, (D–F) and combined αGPIIb/D-arginine vasopressin (DDAVP) treatment (G–I). H&E, magnification 20×.

Fig. 9.

Immunohistochemical staining with monoclonal antibodies against CD41 (platelet marker, red) and DAPI nuclear counterstaining (blue). (A,B) show representative images of an untreated porcine xenocontrol perfusion. (A) shows that platelet deposition occurs in the porcine hepatic tissue within 30 min of initiating xenogeneic perfusion. (B) demonstrates that this deposition increases within the liver by the conclusion of perfusion (360 min). Magnification 20×. (C) shows that although there is less platelet deposition in the allogeneic control during the early phase of perfusion, there is near equivalent IHC CD41 scoring for all control and treatment groups after 360 min of perfusion.