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Synthetic Liver Function is Detectable in Transgenic Porcine Livers Perfused with Human Blood

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Abstract

Background—In addition to immune barriers, molecular incompatibilities between species are predicted to limit pig liver survival in primate xenotransplantation models. Assessment and measurement of synthetic function of genetically modified porcine livers after *ex vivo* perfusion with human blood has not previously been described.

Methods—Eight porcine livers from α 1,3-galactosyl transferase knockout and human membrane cofactor (GalTKO.hCD46), six livers from GalTKO.hCD46 and N-glycolylneuraminic acid knock out (GalTKO.hCD46.Neu5GcKO), and six livers from GalTKO.hCD46 with humanized decay-accelerating factor (hCD55), endothelial protein C receptor (hEPCR), tissue factor pathway inhibitor (hTFPI), and integrin associated protein (hCD47) (GalTKO.hCD46.hCD55.hEPCR.hTFPI.hCD47) pigs were perfused with human blood under physiologic conditions. Timed blood samples were tested for liver enzymes and for pig-specific albumin production via western blot.

Results—Porcine albumin levels increased with time in all experiments. By densitometry, GalTKO.hCD46.Neu5GcKO livers had the highest albumin levels, measured both as total produced, and when controlled for perfusion duration, compared to GalTKO.hCD46 ($p=0.068$) and GalTKO.hCD46.hCD55.hEPCR.hTFPI.hCD47 livers ($p=0.04$).

Conclusions—Porcine livers perfused with human blood demonstrated the synthetic ability to produce albumin in all cases. GalTKO.hCD46.Neu5GcKO pig livers demonstrated the most robust

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Disclosures

David Ayares is the Chief Scientific Officer of Revivacor, Inc. Richard N. Pierson III serves on the Scientific Advisory Board of Revivacor.

Author Contributions

AC participated in concept/design, performance of the research, data analysis, and drafting manuscript. BMF participated in research design and drafting manuscript. JMP participated in performance of the research and data analysis. CP and DA provided essential reagents. NAO and CTL performed critical revision of the paper. RNP, RNB, AMA, and JCL participated in research design, data analysis/interpretation, and critical revision of article.

albumin production. This suggests that the Neu5GcKO phenotype provides a protective effect on the graft due to decreased human antibody recognition and graft injury.

Keywords

liver xenotransplantation; synthetic function; transgenic pigs

Introduction

Recent work in xenotransplantation has focused on addressing immunologic barriers with novel gene manipulations and drug regimens. This has been met with success in terms of longer surviving grafts in preclinical large animals models of heart¹, kidney², and liver³ xenografts. While extending graft survival and minimizing graft injury is important, these longer durations of graft life now mandate looking at graft function.

The liver plays a central role in all metabolic processes in the human body contributing to plasma protein synthesis, including albumin and clotting factors, detoxification, bile production, and glucose regulation. At any given time approximately 25% of the liver's energy is devoted to albumin production, with an average of 12 grams of albumin made daily. Serum albumin plays a critical role in homeostatic functions including maintenance of plasma oncotic pressure, regulation of body fluid distribution, and transport of small molecules⁴. The large synthetic burden is unique to the liver and further highlights the importance of functional assessment of liver xenografts. A 2015 review of cross-species hepatocyte transplants, which included studies examining albumin production, highlighted promising results when wild type pig or human hepatocytes were injected into mice, rats or monkeys⁵. Specifically, a study by Nagata et al, which transplanted wild type porcine hepatocytes into the spleens of cynomolgus monkeys treated with immunosuppression, demonstrated porcine albumin production (measured via ELISA) for up to 80 days⁶. However, a more recent experience using genetically modified pig hepatocytes injected into the spleen, kidney, mesenteric lymph nodes, and adipose tissue of baboons showed disappointingly low levels of porcine mRNA and albumin production⁷. One study looking at orthotopic genetically modified pig liver transplants into baboons was able to show evidence for production of porcine albumin and clotting factors over several days⁸.

New genomic editing techniques now allow for multiple targeted genetic modifications in donor animals (pigs). Addressing or eliminating known xeno-antigens as well as complement and coagulation regulation incompatibilities could protect and enhance the function of porcine liver xenografts. Current genetic modifications available in pigs include knockout of α -1,3 galactosyltransferase enzyme (GalTKO), removing the 1,3 α Gal epitope from the surface of pig cells, a key step in preventing hyperacute rejection⁹. Further attention to addressing "non-Gal" xeno-antigens has resulted in the production of "triple knock out" pigs, which lack N-glycolylneuraminic acid (Neu5Gc) and β 1,4N-acetylgalactosaminyl transferase (β 4GalNT2), both surface carbohydrate moieties, in addition to the GalTKO¹⁰, designed to reduce residual antibody mediated graft injury. Other modifications are knock-ins of genes encoding human complement regulatory proteins such as CD46 (hCD46, membrane cofactor protein) and CD55 (hCD55, decay accelerating

factor); expression of these complement pathway regulatory proteins actively inhibit complement injury to cells on which anti-pig antibody is bound¹¹. Similarly, expression of human endothelial cell protein C receptor (hEPCR) and human tissue factor pathway inhibitor (hTFPI) have been genetically introduced into pigs, intending to inhibit non-physiologic activation of clotting mechanisms that are triggered when pig endothelium is exposed to human blood¹².

While some groups have looked at hepatic function in xenotransplant in vivo models, detection of synthetic function such as albumin was inconsistent, and the ability of human blood to support synthetic function of multi-gene modified pig livers *ex vivo* has not previously been described. Here we investigate the albumin production capability of several strains of genetically modified pig livers when perfused *ex vivo* with human blood.

Materials and Methods

Animals

Twenty transgenic pigs were obtained from Revivicor Inc (Blacksburg, VA) with the following genotypes: α 1,3-galactosyl transferase knock out and human membrane cofactor (GalTKO.hCD46, “2-gene”, n=8), GalTKO.hCD46 and N-glycolylneuraminic acid knock out (GalTKO.hCD46.Neu5GCKO, “3-gene”, n=6), and GalTKO.hCD46 and human decay-accelerating factor (hCD55), endothelial protein C receptor (hEPCR), tissue factor pathway inhibitor (hTFPI), and integrin associated protein (hCD47) (GalTKO.hCD46.hCD55.hEPCR.hTFPI.hCD47, “6-gene”, n=6). Expression of all transgenes in the liver was validated by immunohistochemistry (data not shown). All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Maryland School of Medicine.

Liver procurement

Pig liver procurement has been described previously¹³. Briefly, pigs were placed under inhalation anesthesia (isoflurane 1–4%) and a midline laparotomy was made. The aorta and vena cava were isolated and after systemic heparin was administered (1000IU/kg; Midwest Veterinary supply, Lakeville, MN, USA) the infrarenal aorta was cannulated and the liver flushed with cold UW solution (Organ Recovery Systems, Chicago, IL, USA). The liver was removed in standard clinical fashion and cannulas were placed in the suprahepatic vena cava, celiac artery, portal vein, and common bile duct.

Blood Collection

All protocols were approved by the University of Maryland School of Medicine Institutional Review Board. Human blood was collected from volunteers in citrated blood bags (Terumo Medical Corporation, Somerset, NJ, USA) and used within four hours of collection.

Blood Additives

Collected human whole blood was diluted 1:2 with blood type compatible human fresh frozen plasma (Indiana Blood Center, Indianapolis, IN, USA). Calcium chloride (8.8mmol) (Midwest Veterinary Supply, Lakeville, MN, USA) was added to reverse citrate. All

experiments additionally received heparin (Midwest Veterinary Supply, Lakeville, MN, USA) titrated to ACT > 400 seconds, and prostaglandin I₂ analogue (Remodulin 2mg/hr, United Therapeutics, Silver Spring, MD, USA). In some experiments the blood perfusate additionally included a histamine receptor antagonist (famotidine, 12mg/unit of blood, Midwest Veterinary Supply, Lakeville, MN, USA), a thromboxane synthase inhibitor (1-benzylimidazole, BIA, 120mg/unit of blood, Sigma-Aldrich, St. Louis, MO, USA), and a murine anti-human GPIIb-blocking antibody (6B4) fragment (α GPIIb, 10mg/unit of blood, provided by H. Deckmyn, Laboratory for Thrombosis Research, University of Leuven, Belgium), and the donor pig was pretreated with desmopressin (DDAVP, Diamondback, Scottsdale, AZ, USA) on the day prior to liver harvest (SDC Table 1).

Liver Perfusion

Our liver perfusion system has been previously described¹³. Briefly, blood was placed in a heated reservoir (37°C) and pumped through an oxygenator (Dideco neonatal oxygenator, Sorin Group, Arvada, CO, USA) and the outflow was split and pumped through the hepatic artery and shunted back to the reservoir. Partially oxygenated blood was pumped through the portal vein. Outflow from the liver was returned via the suprahepatic vena cava. Perfusions were terminated when elevated vascular pressures precluded flow or if uncorrectable metabolic derangements were noted. Thus “survival” was calculated as the time from reperfusion of the liver with human blood to the point of termination as dictated by the above criteria.

Blood Sampling and Processing

Blood was collected from the suprahepatic vena cava outflow at timed intervals throughout the perfusion in EDTA vacutainer tubes (Becton Dickinson), supplemented with meclofenamate (10 μ g/mL) and benzamidine (1mM) (Sigma-Aldrich, St. Louis, MO, USA) after the vacuum was broken. The tubes were centrifuged at 2500rpm for 15 minutes. Plasma was stored at -80°C until used. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured via commercial laboratory (Antech, Rockville, MD, USA).

Western Blotting

For each experiment a pre-perfusion sample, final time point sample, and one additional sample (2-hour or 4-hour time point depending on experiment length) were used for western blotting analysis. 2 μ g of total protein for each time point was first denatured in Laemmli loading buffer containing 2- β mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA) before separation on a 4-20% Tris SDS-PAGE gel (Bio-Rad Laboratories, Hercules, CA, USA) and transfer to a nitrocellulose membrane (Invitrogen, Carlsbad, CA, USA). The membrane was blocked with 3% dry milk in PBS buffer (Cell Signaling Technology, Danvers, MA, USA) for 1 hour at room temperature. Membranes were then incubated with the following primary antibodies: goat anti-pig albumin, 1:10,000 dilution, (ab112980, Abcam, Cambridge, MA, USA) and mouse anti-human IgG, 1:10,000 dilution, (05-4200, Thermo Fischer, Rockford, IL, USA) which were diluted in PBS-T-M (PBS+ 0.01% Tween + 0.1% milk), overnight at 4°C. After washing with PBS-T-M, membranes were then incubated with the following secondary antibodies: rabbit anti-goat IgG Alexa Fluor 680 (1:30,000 in PBS-T-M)

(A27020, Thermo Fischer, Rockford, IL, USA) and donkey anti-mouse IgG Alexa Fluor 790 (1:30,000 in PBS-T-M) (A11371, Thermo Fischer, Rockford, IL, USA). Immunofluorescent bands were detected using an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE, USA), and individual band intensities were measured. The presence of a human IgG band (loading control) as well as an albumin band was verified in each lane. The background intensity signal for the albumin band from a human control plasma lane sample was subtracted from all experimental bands on the same gel.

Data Analysis

Band intensities were averaged across genotypes at comparable time points. Average intensity was divided by experimental length to control for differing durations of perfusions. All data is presented as mean and standard error of the mean. Two tailed t-tests were used to compare groups with a p value <0.05 considered significant. All statistical analyses were done with GraphPad Prism software (GraphPad, Inc)

Results

Graft Survival

The mean survival was similar in the 2-gene (397 min, SEM 83, range 115–600 min) and the 3-gene group (293 min, SEM 63, range 125–480 min) ($p=0.39$). Survival was longer in the 6-gene group (517 min, SEM 77, range 283–795 min) which was not significant compared to the 2-gene group ($p=0.29$), and of borderline significance compared to the 3-gene group ($p=0.05$) (Figure 1). Within each group survival did not appear to be affected by drug treatment (Table 1).

Vascular Resistance

In all experiments, the perfusion pressures of both the portal vein and hepatic artery were maintained within normal physiologic ranges until approximately 30 minutes prior to termination when resistance in both vessels sharply increased. This pattern of behavior was observed regardless of drug treatment.

Transaminase Levels

ALT levels increased gradually in all experiments. In the 3-gene and 6-gene groups there was no difference, and ALT levels stayed within normal range (Figure 2A). ALT levels in the 2-gene group increased more sharply after 2 hours, and were higher than the other two groups. However this was due to one single experiment with high transaminase levels for unknown reasons. Controlling for this experiment, there was no difference in ALT levels between the three groups and all values were within physiologic range (Figure 2B). AST levels in the 6-gene group increased gradually and remained within physiologic range. However, AST levels in the 2-gene and 3-gene groups rose sharply to above physiologic normal ranges by one hour (Figure 2C). Again, one experiment in the 2-gene group had high transaminase levels for unknown reasons and controlling for this experiment showed AST levels closer to those of the 3-gene group (Figure 2D).

Albumin Production

All livers were noted to produce porcine albumin, and albumin levels increased over time in all experiments (Figure 3A, 3B). A human IgG band serving as a loading control was detected in addition to the albumin band in all experimental lanes. There was minimal cross reactivity with human albumin, and any background signal measured in human control samples was subtracted from the porcine samples. Total albumin produced, as measured by band intensity (I) of final time point samples in all experiments, was divided by the length of perfusion in hours to control for different durations of perfusion. Average albumin production was highest in the 3-gene group (1515 I/hr), followed by the 2-gene group (576 I/hr), and lowest in the 6-gene group (278 I/hr) (Figure 3C). The 3-gene group produced significantly more albumin than the 6-gene group ($p=0.04$).

Discussion

Extending graft survival in xenotransplantation models is a key step towards clinical application. With longer functioning xenografts it is possible and necessary to assess their function rather than just their resistance to immunologic injury. Few studies have looked at liver xenograft protein production. Ekser et al measured levels of pig-specific proteins produced in baboons who had undergone GalTKO.hCD46 porcine liver transplants⁸. They were able to demonstrate via western blot that over a period of 4-7 days porcine livers produced albumin, fibrinogen, haptoglobin and plasminogen. They also showed that the levels of multiple coagulation factors increased over time pointing to synthesis by the transplanted liver. These encouraging results prompted us to test if human blood could support synthetic function in a clinically applicable model. In our study, we showed that transgenic pig livers were able to carry out synthetic metabolism when perfused ex vivo with human blood. Notably, albumin production was detected after a short time period of a few hours in our experiments, showing evidence of immediate graft function. Total albumin detected increased over time in all experiments, however the actual rate of albumin production decreased over time in the longer experiments. This could reflect an initial robust response to a stress state detected by the liver on reperfusion followed by equilibration to a lower steady state of synthesis over time. We found that the GalTKO.hCD46.Neu5GCKO livers performed better than the GalTKO.hCD46 livers and postulate that this observation is related to the relative protection of the Neu5GcKO from non-Gal antibody mediated injury, based on the model that a more protected liver would be expected to have better function. However, antibody mediated injury is not the only issue affecting liver xenografts. Inflammation, complement activation, and excessive coagulation limit xenograft survival. The 6-gene group had four additional genetic modifications targeting these processes which likely accounted for their longer survival. Thus it seems that regulation of complement and coagulation pathways had a stronger influence on liver survival while protection from immune injury contributed to more robust albumin synthesis.

Within each genotype group there were differences in the drug regimens given. While more livers in the 3-gene and 6-gene groups received the full cocktail of additional pharmacologic interventions, these were added mainly to target platelet activation and sequestration (BIA, aGPIb, DDAVP) phenomena seen in liver xenotransplant models. Based on their

mechanisms of action, these therapies are not expected to have a direct effect on albumin production.

Both AST and ALT levels remained within normal ranges in the 6-gene group. However, the 2-gene and 3-gene groups showed normal ALT levels (when controlling for one experiment) but notably elevated AST levels. This disproportionate rise in AST compared to ALT has been noted before¹⁴. The reason for this is unknown but could be due in part to elevated levels of hemolysis in the 2-gene and 3-gene groups¹³.

The elevated AST levels raise concern for hepatocyte injury, and the possibility that the albumin levels measured reflect release from ruptured cells rather than true albumin production. We feel this is unlikely because albumin is released into the portal circulation as soon as it is manufactured rather than stored in the liver¹⁵, and based on labeling studies in humans, albumin secretion time is short, at about 40 minutes¹⁶. Finally, albumin is synthesized on bound, as opposed to cytoplasmic, ribosomes making it improbable that there is a significant amount free in the cytoplasm of cells¹⁷.

While albumin is a ubiquitous protein made in large amounts by the liver, it is certainly not the only marker of synthetic function necessary to assess in liver xenografts. Consumptive coagulopathy and thrombocytopenia has accounted for early failure of in vivo pig to baboon liver xenotransplantation models^{18,19}. Recently, prolonged survival has been achieved with human coagulation factor supplementation pointing to deficiencies of liver xenograft production or function^{3,20}. Assessment of coagulation factor levels is an important next step in our model.

As important as detecting protein production by the liver xenograft is, we also recognize that ultimately these proteins must themselves be functional. In vitro work by Zhang et al showed that plasma from banna minipig inbreds were capable of activating both the extrinsic and intrinsic coagulation pathways when added to factor deficient human plasma²¹. These results are encouraging that porcine produced proteins would be able to work in both in vivo large animal models and ex vivo pig-to-human models such as ours.

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Abbreviations

αGPIb	anti-human GPIb blocking antibody
ALT	alanine aminotransferase
AST	aspartate aminotransferase
BIA	1-Benzylimidazole
DDAVP	desmopressin

GalTKO	α 1, 3-galactosyl transferase gene knock out
hCD46	humanized membrane cofactor
hCD47	humanized integrin associated protein
hCD55	humanized decay-accelerating factor
hEPCR	humanized endothelial protein C receptor
hTFPI	humanized tissue factor pathway inhibitor
I	Intensity
Neu5GcKO	N-glycolylneuraminic acid knock out
SEM	standard error of the mean

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Figure 1. Survival time

Kaplan-Meier curves showing pig liver survival time for GalTKO.hCD46 (2-gene), GalTKO.hCD46.Neu5GcKO (3-gene), and GalTKO.hCD46.hCD55.hEPCR.hTFPI.hCD47 (6-gene) livers.

Figure 2. Transaminase elaboration

A: ALT levels rose slowly throughout perfusion and remained within normal limits in the GalTKO.hCD46.Neu5GcKO (3-gene) and GalTKO.hCD46.hCD55.hEPCR.hTFPI.hCD47 (6-gene) groups. ALT levels rose more sharply in the GalTKO.hCD46 (2-gene) group. B: When controlling for one experiment in the GalTKO.hCD46 (2-gene) group that had disproportionately high ALT and AST levels for unknown reasons there was no difference in ALT levels between the three groups. C: AST levels remained low in the GalTKO.hCD46.hCD55.hEPCR.hTFPI.hCD47 (6-gene) group. AST levels rose to above physiologically normal levels in the GalTKO.hCD46 (2-gene) and GalTKO.hCD46.Neu5GcKO (3-gene) groups with the highest levels seen in the GalTKO.hCD46 group (2-gene). D: When controlling for one experiment in the GalTKO.hCD46 (2-gene) group that had disproportionately high ALT and AST levels for unknown reasons the AST levels of the GalTKO.hCD46 (2-gene) and GalTKO.hCD46.Neu5GcKO (3-gene) groups were not significantly different.

Figure 3. Albumin production in plasma

A, B: Representative blots of albumin production. First lane showing molecular weight marker (MW). Minimal pig albumin signal was detected in human plasma samples (Human), while pig plasma (Pig) was strongly positive. Albumin bands (red) increased in intensity over time in all experiments. An appropriate IgG loading control band (green) was observed in all experimental lanes. C: Band intensities were measured by densitometry and the background signal detected in human control plasma was subtracted from each time point in the same gel. Total albumin detected was then divided by the duration of perfusion

to account for different lengths of perfusions and obtain intensity/hr units. The GalTKO.hCD46.Neu5GCKO (3-gene) livers had the most robust albumin production.

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Table 1

Experimental Genotypes and Drug Regimens

Genotype	Experiment	Survival (min)	Famotidine, BIA	αGP1b	DDAVP
GalTKO.hCD46	1	115	X	X	
	2	600	X	X	
	3	597	X	X	
	4	202	X	X	
	5	208			
	6	600			X
	7	195			X
	8	600			X
GalTKO.hCD46.Neu5GcKO	9	160			
	10	240			
	11	480			
	12	270			
	13	125	X	X	X
	14	480	X	X	X
GalTKO.hCD46.hCD55.hEPCR.hTFPI.hCD47	15	240	X	X	X
	16	460	X		X
	17	285	X		X
	18	360	X	X	X
	19	660	X		X
	20	795	X	X	X