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Human Immune-reactivity against Liver Sinusoidal Endothelial Cells from GalT α (1,3)GalT-Deficient Pigs

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Abstract

Background—Elimination of galactose- α (1,3)galactose (Gal) expression in pig organs has been previously shown to prevent hyperacute xenograft rejection. However, naturally present antibodies to non-Gal epitopes activate endothelial cells leading to acute humoral xenograft rejection. Still, it is unknown whether xenogeneic pig liver sinusoidal endothelial cells (LSECs) from α (1,3)galactosyltransferase (GalT)-deficient pigs are damaged by antibody and complement-mediated mechanisms. The present study examined the xeno-antibody response of LSECs from (GalT)-deficient and wild pigs.

Methods—Isolated LSEC from wildtype and GalT pigs were exposed to human and baboon sera, IgM and IgG binding was analyzed by flow cytometry. Complement activation (C3a and CH50) was quantified in vitro from serum-exposed LSEC cultures using Enzyme-Linked Immunosorbent Assay. Levels of complement activated cytotoxicity (CAC) were also determined by a fluorescent Live Dead Assay and by the quantification of LDH release.

Results—IgM binding to GalT KO LSECs was significantly lower (80% human and 87% baboon) compared to wildtype pig LSEC. IgG binding was low in all groups. Moreover, complement activation (C3a and CH50) levels released following exposure to human or baboon sera were importantly reduced (42% human and 52% baboon), CAC in GalT KO LSECs was reduced by 60% in human serum and by 72% in baboon serum when compared to wildtype LSECs and LDH release levels were reduced by 37% and 57% respectively.

Conclusions—LSECs from GalT KO pigs exhibit a significant protection to humoral-induced cell damage compared to LSECs from wild pigs when exposed to human serum. Though insufficient to inhibit xenogeneic reactivity completely, transgenic GalT KO expression on pig livers might contribute to a successful application of clinical xenotransplantation in combination with other protective strategies.

Keywords

Xenotransplantation; Liver endothelial cells; GalT α (1,3)GalT-Knockout pigs

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Introduction

End-stage liver failure accounts for over 25,000 deaths/year and is the tenth most frequent cause of death in the United States. The only known cure for liver failure is orthotopic liver transplantation, but unfortunately less than 7000 organs are available per year in the United States. It is estimated that due to the high prevalence of Hepatitis C Virus infection in the population (about 3%) this ratio will get significantly worse over the next few decades. Organ or cell xenotransplantation is one attractive strategy to meet the increasing organ demand for human transplantation (2,15). However, transplantation of porcine organs into primates normally initiates a cascade of events that starts with binding of naturally occurring antibodies to xenogeneic epitopes on the endothelium and results in hyperacute xenograft rejection (HXR) within minutes to hours of transplantation (6,10,17,19). These xenoreactive antibodies predominantly recognize galactose- $\alpha(1,3)$ galactose (Gal), a terminal disaccharide synthesized by $\alpha(1,3)$ galactosyltransferase (GalT) on the endothelial surface of pigs followed by classical pathway activation of the recipient's complement system (9,16,17). The formation of these components is accompanied by perturbation of endothelial cell function and morphology, which results in loss of vascular integrity and development of edema, hemorrhages and thrombi formation.

Previous studies using organs from animals that lacked both alleles of the gene to express Gal showed that HXR was prevented in pig-to-primate models of kidney and heart transplantation (13,20). However, GalT KO organs are still rejected in weeks to months by acute humoral xenograft rejection (AHXR), which represents the next major hurdle in pig-to-primate xenotransplantation. AHXR, also named acute vascular rejection, is characterized by the activation of microvascular endothelial cells leading to thrombotic microangiopathy. How exactly the endothelial cells become activated is unknown, but activity of antibodies directed against non-GalT antigens are thought to play an important role (5). Thus, the development of GalT knockout (KO) pigs could represent a contributing solution to cell therapy or organ xenotransplantation.

Recent studies have demonstrated that isolated aortic endothelial cells demonstrated 33% to 14% of the activity of human natural antibodies remained even the GalT epitope has been eliminated (1). At the same time, over 20% of complement activated cytotoxicity (CAC) remained, indicating that a considerable amount of humoral immune reactions directed against non-GalT antigens remain even in GalT KO conditions. These recent studies raise important questions regarding the compatibility of Gal-KO organs for liver cell xenotransplantation.

It is well known that hepatocytes present their antigens to the systemic circulation by sending processes through the fenestrations of the liver sinusoidal endothelial cells (LSECs) (8), exhibiting increased resistance to CAC (11). Recent studies applied human serum, as a source of xenoreactive natural antibodies and complement, to porcine endothelium and demonstrated that complement deposited on porcine endothelial cells is responsible for the early adhesion and transmigration of leukocytes (1). Porcine LSECs, the main antigenic component of liver allografts, bind significantly lower levels of human natural antibodies compared to aortic endothelial cells (3). These observations raise the possibility that the liver has a unique position in xenotransplantation. Therefore, it is important to know whether LSECs from GalT KO pigs exhibit a resistance to mechanisms of humoral xenograft rejection. In this study, we quantified components of humoral xenograft rejection in human and baboon serum against the microvasculature of the liver *in vitro* in order to identify new potential therapeutic targets or cell combinations necessary for inhibition of acute cell xenograft rejection. We looked at the ability of the human natural antibody binding and complement activity in both wildtype and GalT KO LSECs.

Materials and Methods

LSEC Isolation

LSECs were isolated from the livers of SLA^{dd}, GalT^{+/+} miniature swine (wildtype), and partially inbred, SLA^{dd}, GalT^{-/-} (GalT-KO) miniature swine, kindly provided by Dr David H. Sachs. Animals were kept under standard conditions and cared for in accordance with the guidelines set forth by the Committee on Laboratory Resources, National Institutes of Health. The liver was excised and put on ice. After cannulation of the portal vein branch to the left lateral lobe, digestion of the tissue was achieved using a two step-perfusion method. First, the lobe was flushed for 10 minutes with ice-cold 0.9% NaCl solution supplemented with 5% dextrose, 20 U/L heparin and 4 meq/L KCl. Subsequently, the lobe was perfused for 18 minutes at 37 °C with a solution of 0.5% collagenase type IV (Sigma Aldrich, St Louis, MO) in Krebs Ringer Buffer supplemented with 3 mM CaCl₂. The lobe, weighing between 175 and 225 grams, was then cut into 12 pieces with a scissor and the liver cells were gently dispersed into Krebs Ringer Buffer on ice.

After collection of a total of 400 ml of cell suspension, clumps were removed by filtration through a 250 µm and then a 150 µm mesh. Hepatocytes were pelleted by a differential centrifugation step at 50g for 10 minutes at 4 °C. The non-parenchymal cells, present in the supernatant, were collected by centrifugation at 300g for 15 minutes at room temperature. Cells were resuspended in 30 ml of elutriation buffer that consisted of Gay's Balanced Salt Solution supplemented with 0.1% bovine serum albumin. DNA-se was added (10 µg/ml) and the cell suspension incubated at 37 °C for 15 minutes. All subsequent steps were performed at room temperature. Differential elutriation was performed using an elutriating centrifuge with a JE-5.0 rotor (Beckman Coulter, Fullerton, CA) using a standard chamber at 2,500 rpm. The suspension was introduced at a flow rate of 18 ml/min. This flow-rate was maintained for 15 minutes. LSECs were collected at a flow rate of 38 ml/min. The cells were pelleted at 300g for 15 min and resuspended in microvascular endothelial growth medium EGM-2-MV (Cambrex, East Rutherford, NJ). A differential adhesion step was performed for 5 minutes before non-adhering cells were counted and viability was assessed by trypan blue exclusion. The yield was routinely 15–40 × 10⁶ cells with viability exceeding 90%.

LSEC Culture & Characterization

Cells were cultured at a density of 5 × 10⁵ cells/cm² at 37 °C in a humidified 5% CO₂ incubator in 12-well plates that had previously been coated with human fibronectin (R&D Systems, Minneapolis, MN) at 50 µg/ml for 1 hour. LSEC purity was greater than 85% as assessed by FITC labeled acetylated-LDL (Invitrogen, Carlsbad, CA) uptake and the lack of stellate cell autofluorescence. Cells were cultured for 2 days before complement activation studies.

Human and Baboon Sera

Human and baboon serum were collected from normal healthy donors and stored at –80 °C until use. Complement was inactivated in selected samples by incubation at 56 °C for 60 minutes. Care of animals was in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health. The institutional subcommittee on research animal care approved the protocols used in this report. An informed written consent was obtained from each healthy donor before the procedure.

Antibody Binding

Freshly isolated wildtype or GalT KO LSECs (2 × 10⁵) were incubated in human or baboon serum diluted at a 1:2, 1:20 or 1:200 ratio with PBS for 30 min at 37 °C. Cells were washed

and collected by centrifugation at 300g for 15 minutes. Binding of IgM and IgG was detected by incubation with a FITC-labeled swine anti-human IgM antibody and a TRITC-labeled swine anti-human IgG antibody (Accurate Chemical Scientific Corporation, the Netherlands) at a 1:100 dilution for 30 minutes on ice. Binding was quantified by flow cytometry using a FACSCalibur (BD, San Jose, CA), analyzing 10,000 events per experiment. Experiments were performed with two separate batches of cells for GalT KO and wildtype cells.

Complement Activation

LSECs were incubated with human or baboon serum diluted 1:2 with PBS for 60 minutes at 37 °C. Supernatants were collected and stored at -80 °C for ELISA. Analysis of C3a anaphylotoxin levels, a non-specific measure of complement activation, was performed using a commercially available kit (BD, San Diego, CA) per manufacturer's instructions. The CH50 Eq Enzyme Immunoassay (Quidel, San Diego, CA) was performed according to the vendor's instructions to quantify the total classical complement activity. Experiments were performed in triplicates and performed with two separate batches of cells for each condition.

Complement Activated Cytotoxicity

Complement activated cytotoxicity after incubation with human or baboon serum was assessed using a fluorescent Live-Dead Viability Assay (Molecular Probes, Eugene, OR) according to the vendor's instructions. In this assay, the cytoplasm of live cells accumulates green fluorescent calcein due to esterase activity, while the nucleus of dead cells is labeled fluorescently red by ethidium homodimer due to loss of membrane integrity. Incubation with heat-inactivated serum was used as a negative control. Live and dead cells were captured on a Zeiss 200 Axiovert microscope and quantified in 4 random images per well using the public software ImageJ (<http://rsb.info.nih.gov/ij/>). Lactate dehydrogenase (LDH) release in supernatants of the serum-exposed cultures was quantified using the LDH Cytotoxicity Assay Kit II (Biovision, Mountain View, CA) according to the manufacturer's instructions. Experiments were performed in triplicates and performed with two separate batches of cells for each condition.

Statistical Analysis

Data is expressed as the mean $\pm$ standard deviation. Statistical significance was determined by a two-tailed Student's t-test. A *P*-value of 0.05 was considered statistically significant.

Results

Antibody Binding

Binding of preformed xenoreactive antibodies to porcine cell surface antigens is an essential step in both HXR and AHXR. To assess the decrease in preformed antibody binding to pig LSECs due to the elimination of the GalT epitope, we analyzed IgM and IgG binding to wildtype and GalT KO pig LSECs using FACS. Significant levels of human and baboon serum preformed IgM binding to LSECs isolated from wildtype pigs were observed. For human serum, IgM reactivity decreased in GalT KO LSECs by 74%, 81% and 84% at the different serum dilutions used. Decreases were 82% 85% and 94% for baboon serum IgM reactivity (fig. 1a/b). Levels of IgG binding to pig LSECs was low in both human and baboon serum (fig. 1c/d). Only with 50% serum, a 27% reduction in the level of human IgG binding was observed. No difference was detected at lower serum concentrations and no changes in IgG binding were measured for baboon serum. These results demonstrate that the majority of IgM binding to pig LSECs is prevented in GalT KO animals and that levels of preformed IgG binding are less prominent and only modestly affected by knocking out GalT.

Complement Activation

Levels of complement activation in serum samples exposed to wildtype and GalT KO LSECs was assessed using two different assays. Levels of C3a complement were measured by an enzyme-linked immunosorbent assay as an indication of total complement activation (fig. 2a). C3a levels were reduced by 42% ($P < 0.05$) in the serum of pig GalT KO LSECs compared to cultured wildtype LSECs. The CH50 assay (fig. 2b) measures the classical complement pathway demonstrated a 52% ($P < 0.005$) reduction in activity in the serum of pig GalT KO LSECs lacking expression of GalT antigens.

Complement Activated Cytotoxicity (CAC)

To determine whether pig LSECs are susceptible to CAC and to what degree this cytotoxicity is retained for LSECs when GalT expression is blocked, viability and LDH release were measured in pig LSEC following one hour exposure to human or baboon serum. A considerable quantity of dead cells were observed when pig LSECs were exposed to normal human plasma (fig. 3a). In contrast, almost no cells died after heat-inactivation of complement in the serum samples (fig. 3b), demonstrating that pig LSECs are susceptible to CAC in human serum. Significant decrease in the cytotoxicity levels was measured for LSECs isolated from GalT KO pigs when compared to wildtype cells (fig. 3c). The GalT KO LSEC demonstrated a 60% lower cell death following exposure to human serum ($P < 0.001$) and a 72% drop following exposure to baboon serum ($P < 0.001$). Levels of LDH release from GalT KO LSECs were reduced by 37% and 57% respectively (fig. 3d; $P < 0.001$). These results suggest that LSECs derived from GalT KO still showed a considerable amount of cytotoxicity after exposure to primate serum.

Discussion

A major obstacle in the transplantation of porcine organs to humans is the presence of naturally occurring antibodies to xeno-antigens expressed on the endothelial surface of the blood vessels in the transplanted organ. With the availability of GalT KO pigs, much of the attention has been shifted to non-GalT epitopes. In pig-to-primate kidney transplantations using organs from GalT KO animals, a strong association between anti-non GalT antibodies and AHXR was observed (14). The blood of every adult naturally contains anti-non GalT antibodies, such as IgM's and IgG's against N-glucolylneuraminic acid (18), an antigen expressed on porcine endothelial cells but not in humans. On the other hand, it has been demonstrated that porcine LSECs bind significantly lower levels of human natural antibodies compared to aortic endothelial cells (15). Therefore, we investigated whether the LSEC isolated from GalT deficient pigs maintain an immunoprivileged status when exposed to human or baboon plasma. We used an in vitro cell culture model to study the interaction of human serum with porcine endothelial cells and showed that human serum, as a source of xenoreactive antibodies and complement, can directly activate porcine endothelium.

We demonstrate herein that LSECs derived from GalT KO pigs are less susceptible to mechanisms of humoral xenograft rejection than wildtype LSECs, although certain levels of antibody binding remain even in the absence of the GalT epitope. Baumann and colleagues reported similar reductions in levels of IgM binding in GalT KO cells for aortic endothelial cells (1). They showed that generation of Gal-deficient pigs has overcome hyperacute anti-Gal-mediated xenograft rejection in nonhuman primates. However, non-Gal anti-porcine NAb represent a potentially relevant immunological hurdle in a subgroup of individuals by inducing endothelial damage in xenografts.

There are different strategies to circumvent HXR and AHXR, including the use of organs from pigs transgenic for complement-regulating factors such as CD55 (human decay-accelerating

factor, hDAF), CD46 membrane cofactor protein or CD59. Kidneys and hearts from hDAF transgenic pigs transplanted into NHP have shown similar results in rejection prevention when compared to GalT knockout organs (4,7,12). Another strategy is to knockout the gene responsible for important non-GalT epitope expression such as N-glucolylneuraminic acid (18), but this genotype has yet to be created in pigs. Our results suggest that elimination of GalT antigens may have to be combined with one or more of these strategies to render pig liver cells resilient to humoral cytotoxic effects of primate serum. Although pig-to-primate orthotopic or liver cell transplantation remains the ultimate test for organ rejection, our experiments provide valuable information about the advantageous immune reactivity of LSECs isolated from GalT KO pigs to human and baboon serum in vitro that can be use in future experiments to evaluate approaches necessary to determine the best route toward clinical organ/cell xenotransplantation.

In conclusion, our data shows that LSECs from GalT KO pigs provide an in vitro protective role to heterologous humoral mediated damage, but they also suggest that the extent of protection is limited. The results in this report may be relevant for designing future therapeutic strategies aimed at prolonging xenograft survival.

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Figure 1. IgM and IgG reactivity of human and baboon serum to pig LSECs from wildtype and Gal KO animals

LSECs were isolated and exposed to varying concentrations of human and baboon serum. Binding of IgM and IgG antibodies was detected by the binding of a fluorescently-labeled secondary antibody. Analysis was performed using flow cytometry. Data is shown as mean $\pm$ standard deviation. LSEC, liver sinusoidal endothelial cell; Gal KO, $\alpha(1,3)$ galactosyltransferase knockout.

Figure 2. C3a and CH50 complement activity levels in human serum exposed to pig LSEC from wildtype and GalT KO animals

LSECs were cultured for 2 days before exposure to human serum. Samples were collected and analyzed for C3a levels and CH50 activity using ELISA. Data shown are mean $\pm$ standard deviation of two separate experiments performed in triplicate. * $P < 0.05$, ** $P < 0.005$ (Student's t-test). LSEC, liver sinusoidal endothelial cell; Gal KO, $\alpha(1,3)$ galactosyltransferase knockout.

Figure 3. CAC in wildtype and GalT KO pig LSECs by exposure to human and baboon serum
Cultured LSECs were exposed to (A) normal and (B) heat-inactivated human serum. Live (green) and dead (red) cells were visualized using a fluorescent Live Dead Assay. (C) Quantification of CAC by image analysis. (D) LDH release. Data shown are mean $\pm$ standard deviation of two separate experiments performed in triplicate. CAC, complement activated cytotoxicity; GalT KO, $\alpha(1,3)$ galactosyltransferase knockout; LSEC, liver sinusoidal endothelial cell. Scale bar 20 μ m.