Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.JournalofSurgicalResearch.com

The fate of human platelets exposed to porcine renal endothelium: a single-pass model of platelet uptake in domestic and genetically modified porcine organs

*James R. Butler, MD, Gregory R. Martens, MD, Ping Li, PhD, Zheng-Yu Wang, MD, PhD, Jose L. Estrada, PhD, DVM, Joseph M. Ladowski, MA, Matt Tector, PhD, and A. Joseph Tector, MD, PhD**

Department of Surgery, Indiana University School of Medicine, Indianapolis, Indiana

ARTICLE INFO

Article history:
Received 22 May 2015
Received in revised form
13 July 2015
Accepted 19 August 2015
Available online 28 August 2015

Keywords:
Platelets
Thrombocytopenia
Kidney
Xenotransplantation

ABSTRACT

Background: Thrombocytopenia may represent a significant challenge to the clinical application of solid-organ xenotransplantation. When studied in a pig-to-primate model, consumptive coagulopathy has challenged renal xenografts. New strategies of genetic manipulation have altered porcine carbohydrate profiles to significantly reduce human antibody binding to pig cells. As this process continues to eliminate immunologic barriers to clinical xenotransplantation, the relationship between human platelets and pig organs must be considered.

Methods: Genetically modified pigs that were created by the CRISPR/Cas9 system with α-1,3-galactosyltransferase (GGTA1)$^{-/-}$ or GGTA1$^{-/-}$ cytidine monophosphate-N-acetylneuraminic acid hydroxylase$^{-/-}$ phenotype, as well as domestic pigs, were used in this study. Autologous porcine platelets were isolated from donor animal blood collection, and human platelets were obtained from a blood bank. Platelets were fluorescently labeled and in a single-pass model, human, or autologous platelets were perfused through porcine organs at a constant concentration and controlled temperature. Platelet uptake was measured by sampling venous output and measuring sample florescence against input florescence. *In vitro* study of the interaction between human platelets and porcine endothelial cells was accomplished by immunohistochemical stain and confocal microscopy.

Results: Differences between human and autologous platelet loss through the porcine kidney were not significant in any genetic background tested (WT $P = 0.15$, GGTA1$^{-/-}$ $P = 0.12$, GGTA1$^{-/-}$ cytidine monophosphate-N-acetylneuraminic acid hydroxylase$^{-/-}$ $P = 0.25$). The unmodified porcine liver consumed human platelets in a single-pass model of platelet perfusion in fewer than 10 min. WT suprahepatic inferior vena cava fluoresce reached a maximum of 76% of input fluoresce within the human platelet cohort and was significantly lower than the autologous platelet control cohort ($P = 0.001$). Confocal microscopic analysis did not demonstrate a significant association between human platelets and porcine renal endothelial cells compared with porcine liver endothelial positive controls.

Conclusions: Our results suggest that in the absence of immunologic injury, human platelets respond in a variable fashion to organ-specific porcine endothelial surfaces. Human

* *Corresponding author*. Department of Surgery, Indiana University School of Medicine, UH 4601, 550 N. University Blvd, Indianapolis, IN 46202. Tel.: (317) 274-4370; fax: 317-968-1497.
E-mail address: jtector@iupui.edu (A.J. Tector).
0022-4804/$ – see front matter
http://dx.doi.org/10.1016/j.jss.2015.08.034

platelets are not removed from circulation by exposure to porcine renal endothelium but are removed by unmodified porcine hepatic endothelium. Kidneys possessing genetic modifications currently relevant to clinical xenotransplantation failed to consume human platelets in an isolated single-pass model. Human platelets did not exhibit significant binding to renal endothelial cells by *in vitro* assay.

1. Introduction

Xenotransplantation using genetically modified pig organs could eliminate the shortage of donor organs for transplantation. Antibody-mediated rejection (AMR) has made it impossible to move to the clinic with xenotransplantation. Two important xenoantigens to which humans have xenoreactive antibodies include gal-α-(1,3)-gal and N-glycolylneuraminic acid (Neu5Gc). These xenoantigens are produced by enzymes that are present in humans and pigs, but in humans, gene activity has been silenced during the course of evolution. α-1,3-Galactosyltransferase (GGTA1) produces the Gal α(1,3) Gal epitope, and the development of GGTA1$^{-/-}$ pigs eliminated 70%–85% of the xenoreactive antibodies that humans have as a barrier to xenotransplantation. The cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH) gene produces Neu5Gc. The creation of GGTA1$^{-/-}$ CMAH$^{-/-}$ pigs has reduced human antibody binding to the point where the crossmatch of pigs with humans is more favorable than a primate-to-human crossmatch [1]. More recently, the creation of GGTA1$^{-/-}$ CMAH$^{-/-}$ B4GalNT2$^{-/-}$ pigs have reduced levels of antibody binding to the point that 80% of patients on transplant lists would not be expected to have early AMR. As current models of pig-to-human xenotransplantation have reached immunologic equivalence to allografts, it is now important to carefully consider potential organ-specific barriers to transplantation.

The most well-characterized organ-specific challenge to xenotransplantation is thrombotic microangiopathy (TMA). This phenomenon is organ dependent as it ranges from graft-limiting TMA in kidney models to consumptive coagulopathy in a liver model of pig-to-primate xenotransplantation. Porcine GGTA1$^{-/-}$ kidneys transplanted into nonhuman primates are not hyperacutely rejected, but they do still fail because of AMR. The AMR seen in GGTA1$^{-/-}$ -to-primate transplantation is most notably characterized by TMA [2]. However, it remains unclear whether the TMA seen in GGTA1$^{-/-}$ -to-primate xenotransplantation occurs because of AMR or is due to coagulation dysregulation based on species incompatibilities in thromboregulatory pathways. As previously described by Burlak *et al.*, an unmodified porcine liver retains the capacity to consume human platelets in the absence of immunologic injury; this raises the possibility of an aberrant relationship between the human platelet and porcine endothelial surface. Although the mechanism of this interaction remains unclear, there is significant data that suggest pig von Willebrand factor (vWf) may aggregate human platelets in the absence of immune-mediated injury or shear force [3]. As shown in Figure 1, differing glycosylation patterns between pig and human vWF A1 domain may affect the affinity of human platelet GpIba receptor.

Although it is clear that graft modifications will be necessary to circumvent the hypercoagulable interaction between porcine liver endothelium and human platelets, no information has existed regarding the porcine renal endothelium and human platelets. The recent success of pig-to-primate renal transplants for several months without coagulopathy has suggested that unlike the porcine liver, the porcine renal endothelium is relatively inert in the absence of immune injury [4,5]. As xenotransplantation continues to move toward clinical application, it is important to move beyond primate models whenever possible, and to specifically consider pig-to-human interaction; this represents the first study to characterize the interaction between human platelets and the porcine kidney. The experiments in this report evaluated whether human platelets perfused through pig kidneys are retained in the absence of an immunologic injury. The results have important implications for the direction of further genetic modification of donor pigs.

2. Methods

2.1. Creation of genetically modified pigs

Cas9 nuclease–mediated gene editing with multiple guide RNAs was used for the efficient production of genetically modified pigs. As previously described, CRISPR Guide RNAs were designed to target the *CMAH* and *GGTA1* genes, responsible for the production of known xenoantigens, Neu5Gc and aGal [6]. Porcine liver–derived cells were cotransfected with these CRISPR/Cas9 constructs using the Neon transfection system (Life Technologies, Grand Island, NY). After the IB4 counter selection, cells bearing CMAH and GGTA1 mutations were enriched and further confirmed by DNA sequencing analysis. These cells were used as nuclear donors for somatic cell nuclear transfer to achieve pregnancy in a surrogate mother [7]. All the animals used in this study were approved by the Institutional Biosafety and Institutional Animal Care and Use Committee of IUPUI.

2.2. Isolation and staining of autologous (donor animal) platelets for perfusion

A total of 500 mL of porcine blood was collected into tubes containing acid-citrate-dextrose at the time of surgery. After centrifugation (5 min at 2000*g*), the top two-thirds of platelet-rich plasma was removed and centrifuged (5 min at 5000*g*). The pellet was resuspended in phosphate-buffered saline (PBS)/acid citrate dextrose 50:1 and washed twice. 10^{10} platelets were obtained by count on hemocytometer, of which 2.5^{10} were labeled with carboxyfluorescein succinimidyl ester