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A Human-specific Mutation Limits Nonhuman Primate Efficacy in Preclinical Xenotransplantation Studies

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Abstract

Background—Patients diagnosed with fulminant hepatic failure face high mortality rates. A potential therapeutic approach for these patients is the use of extracorporeal porcine liver perfusion, to serve as a form of “liver dialysis”. Previously, our laboratory has shown that, during a 72 hour extracorporeal perfusion with human blood, porcine Kupffer cells bind to and phagocytose human erythrocytes causing the hematocrit to fall to 2.5% of the original value. Subsequently, erythrocyte binding has been shown to involve N-acetylneuraminic acid (Neu5Ac) on the surface of human erythrocytes and sialoadhesin on the surface of the porcine Kupffer cells.

Methods—Given that no primate other than the human is known to express the majority of its sialic acid as Neu5Ac, we evaluated whether nonhuman primates would provide adequate evaluation of the loss of erythrocytes that might be expected in a clinical trial of extracorporeal porcine liver perfusion.

Results—We found that while porcine macrophages readily bound human erythrocytes, binding of nonhuman primate erythrocytes was significantly reduced ($p < 0.001$).

Conclusions—This study suggests that nonhuman primates may fail to serve as an adequate model for studying extracorporeal porcine liver perfusion, due to the fact that porcine macrophages do not bind nonhuman primate erythrocytes.

Keywords

xenograft rejection; macrophages; sialic acid and nonhuman primates

Introduction

Xenotransplantation offers the possibility of supplying sufficient organs to meet current transplantation demands. Given the scarce organ supply and the rapid progression of

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Conflicts of Interest:

The authors have no conflicts of interest to disclose

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fulminant hepatic failure, one approach considered as an early clinical application of xenotransplantation is extracorporeal porcine liver xenoperfusion (1). Extracorporeal porcine liver perfusion could serve as “bridge therapy” for patients in liver failure awaiting liver transplantation. To date, several porcine anti-human graft-vs.-host responses have been identified involving the destruction of human platelets and erythrocytes, which limit the use of this therapy (2, 3).

Experiments investigating extracorporeal liver xenoperfusion have spanned nearly 50 years, and almost without exception, investigators have noted significant thrombocytopenia occurring very shortly after the initiation of liver xenoperfusion (4–6). Platelet counts rapidly fall following reperfusion of dog-to-pig liver xenografts, and in pig-to-nonhuman primate liver transplantation, nearly all platelets are removed from the circulation within 1 hour (4, 6). Extracorporeal perfusion of whole human blood through porcine livers leads to near complete loss of platelets within minutes (5). Burlak et al. have recently shown that the mechanism of human platelet destruction by porcine livers involves direct phagocytosis by both Kupffer cells and liver sinusoidal endothelial cells (3). Several investigators have now observed disseminated intravascular coagulation with thrombocytopenia following xenotransplantation (7–10). As clinical support of patients in hepatic failure using extracorporeal porcine livers has been limited to durations of 6–8 hours, and the most significant coagulopathies have been observed only after the xenograft has been engrafted for 48 hours, this problem has not yet been fully evaluated in the pig-to-human combination.

During a 72-hour *ex vivo* perfusion with isolated human blood, porcine livers consume the equivalent of three units of erythrocytes (5). This anti-human erythrocyte reaction is mediated by porcine Kupffer cells (resident liver macrophages) utilizing the lectin sialoadhesin to bind the carbohydrate N-acetylneuraminic acid (Neu5Ac) on the surface of human erythrocytes (11–16). Although more than 32 natural glycoforms of sialic acid are known (17), two main forms are found in mammals, Neu5Ac and N-glycolylneuraminic acid (Neu5Gc) (18). These two glycoforms differ from one another by a single hydroxyl group, a difference produced by the activity of cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH, Fig. 1B) (19–22). This subtle distinction between Neu5Ac and Neu5Gc brings to light a rare genotypic difference between humans and all other nonhuman primates. Due to an *Alu*-mediated deletion in the CMAH gene, which took place sometime after the divergence of the *Homo Pan* genera (Fig. 1A), humans do not express CMAH and thus express the Neu5Ac form of sialic acid to the exclusion of Neu5Gc (19–22). On the contrary, great apes, as well as all other primates excluding humans, have retained the ability to hydroxylate the Neu5Ac precursor. As a result, the majority of the sialic acid expressed on nonhuman primate cell surfaces is expressed as the Neu5Gc form (23).

Lutz et al. recently produced double knockout pigs deficient in both Neu5Gc and galactose α -1,3-galactose (Gal α -1,3-Gal), and demonstrated a significant reduction in the human anti-pig immune response to cells from these animals as compared with cells derived from Gal α -1,3-Gal single knockouts (24). Given that humans cannot synthesize Neu5Gc and are known to have a significant natural antibody response directed against Neu5Gc, it is not surprising that removing Neu5Gc from the surface of porcine cells would reduce the human immune response directed against Neu5Gc-deficient porcine cells. But this research has a

further implication. If one wished to use an animal model to examine the benefit of removing Neu5Gc that might be expected in the pig-to-human model, one would require an animal model in which the recipient animal lacked Neu5Gc-expression and had an inherent humoral response directed against Neu5Gc. Such a model does not exist. As humans are the only mammal deficient in CMAH, thus lacking Neu5Gc expression, no large animal model would be expected to recapitulate the human anti-Neu5Gc response. Thus, it is unlikely that any nonhuman primate model will demonstrate the benefit that might be expected from a CMAH-deficient porcine organ when transplanted into a human recipient.

As a means of testing this inability to evaluate the benefit of CMAH-deficient pigs in any model other than pig-to-human, we have exploited the fact that porcine macrophages bind to and destroy human erythrocytes through a mechanism dependent on Neu5Ac expression (12). We hypothesized that porcine macrophages would fail to recognize nonhuman primate erythrocytes because, like pigs, nonhuman primates express the majority of their sialic acid in the Neu5Gc form. To test this hypothesis, we performed a series of *in vitro* assays assessing the ability of porcine macrophages to bind erythrocytes from various nonhuman primates. The data suggests that the difference in sialic acid expression present when the species combination is pig and human, and not present when the combination is pig and nonhuman primate, limits the ability of porcine macrophages to bind nonhuman primate erythrocytes. We propose that this limitation of the nonhuman primate erythrocytes to provide useful information about what porcine Kupffer cells will do to human erythrocytes is harbinger of the lack of efficacy of nonhuman primates to serve as an appropriate pre-clinical model for either extracorporeal porcine liver xenoperfusion or the evaluation of CMAH/Gal α -1,3-Gal double knockout porcine xenografts.

Results

Porcine macrophages bind human erythrocytes but not chimpanzee erythrocytes

First, given their genetic proximity to humans (Fig. 1A), we reasoned that erythrocytes from a chimpanzee would serve as the best model for determining whether the retained CMAH function seen in nonhuman primates decreased their susceptibility to porcine macrophages. To test porcine macrophage recognition of chimpanzee erythrocytes, primary porcine macrophages previously shown to have high sialoadhesin expression (data not shown), were co-incubated with chimpanzee erythrocytes; binding was quantified using a colorimetric rosetting assay. Human and autologous porcine erythrocytes were used as controls. Previous data collected in our laboratory demonstrate that approximately 70–80 percent of cultured porcine macrophages bind human erythrocytes (15). Compared to porcine macrophage recognition of human erythrocytes (100%), the percent binding of chimpanzee erythrocytes was significantly reduced (13.4%, $p < 0.001$) (Fig. 1C). Of note, porcine macrophages bound autologous erythrocytes (7.1%) to a similar extent as xenogeneic chimpanzee erythrocytes (13.4%). Light microscopy of co-cultured cells confirmed this observation (Fig. 1D).

Porcine macrophage binding of erythrocytes from commonly used primate pre-clinical models is reduced as compared to porcine macrophage binding of human erythrocytes

Next, we evaluated if erythrocytes from other nonhuman primates likely to be used in pre-clinical trials of extracorporeal porcine liver perfusion showed a lack of binding by porcine macrophages as compared to human erythrocytes. Baboon erythrocyte binding (9.1%), Cynomolgus monkey erythrocyte binding (15.2%), and Rhesus macaque erythrocyte binding (18.5%) was significantly less than the binding of human erythrocytes (100%, $p < 0.001$) (Fig. 2A,B,C).

Porcine macrophages fail to recognize nonhuman primate erythrocytes

Given that neither erythrocytes from the chimpanzee, nor erythrocytes from nonhuman primate species commonly used in pre-clinical research were bound by porcine macrophages, we determined if this phenomenon was consistent across a wider array of primate species. We tested porcine macrophage binding of erythrocytes derived from five additional nonhuman primate species representing two different super families, *Hominoidea* and *Cercopithecoidea*. In no case did porcine macrophages bind nonhuman primate erythrocytes to the same degree to which they recognize human erythrocytes, $p < 0.001$ (Fig. 2D).

Discussion

Extracorporeal porcine liver xenoperfusion as a treatment for fulminant hepatic failure has been suggested as a reasonable first step in the clinical evaluation of xenotransplantation. This approach has been advocated for multiple reasons: 1) the shortage of human livers available for transplantation; 2) the high mortality rate associated with fulminant hepatic failure; 3) the lack of a means of temporary support for hepatic failure; 4) that exposure to the porcine organ will be limited to a few days rather than permanent implantation; and 5) long-term immunosuppression may be avoided if the patient is “bridged” to recovery. Although pre-clinical animal studies sometimes fail to accurately predict human responses (25), current FDA requirements for xenotransplantation to advance to clinical trial include evidence for efficacy of the therapy using nonhuman primates in a pre-clinical setting (26). The data in this study provide evidence from nine different nonhuman primate species, including the three most commonly used in pre-clinical studies, suggesting that nonhuman primates will not recapitulate the graft-vs.-host response seen when human blood is perfused through a porcine liver. In addition, Lutz et al. recently produced double knockout pigs deficient in both Neu5Gc and galactose α -1,3-galactose (Gal α -1,3-Gal), and demonstrated a significant reduction in the human anti-pig immune response to cells from these animals as compared with cells derived from Gal α -1,3-Gal single knockouts (24). Data in this study, along with data published by Lutz et al., suggest that, as a result of retained CMAH activity and Neu5Gc expression, nonhuman primates will fail as a model to evaluate the graft-vs.-host response seen when human blood is perfused through a porcine liver as in extracorporeal porcine liver perfusion; and will fail to illustrate the benefit of eliminating Neu5Gc in the host-vs.-graft response seen in pig-to-human whole organ xenotransplantation.

This would not be the first instance where nonhuman primates failed to predict clinical outcomes in transplantation medicine. For example, sirolimus administered at a clinically relevant dose, was found to cause profound gastrointestinal toxicity in rhesus macaques, that viewed in isolation, would have suggested sirolimus would not be tolerated in humans (27). Furthermore, the primate model was unsuccessful at predicting efficacy of anti-CD40 ligand therapy in early pre-clinical studies given the subsequent platelet effects observed in humans (28, 29). While most new therapies in transplantation are studied for safety and efficacy in at least one large animal model prior to phase one studies in humans, studies in large animal models should be designed with the limitations of these models in mind (30).

This study offers insight into a potential pitfall of the use of nonhuman primates in preclinical studies of extracorporeal porcine liver perfusion. The data also allude to the inadequacy of nonhuman primates to demonstrate the benefit that might be expected from a CMAH-deficient porcine organ when transplanted into a human recipient. Given the data presented in the present study and by Lutz et al. (24), as a precondition for clinical trials, we propose that the efficacy of extracorporeal porcine liver perfusion and transplantation of porcine organs from Neu5Gc/Gal α -1,3-Gal double knockouts be evaluated in brain-dead patients determined not to be suitable candidates for organ donation, rather than in nonhuman primates. The concept of using brain dead individuals as research subjects has been called a variety of terms in the literature, but consensus appears to be evolving that such research be referred to as “research on the recently dead” (31). Not surprisingly, research of this nature has been controversial (32–34). However, consensus panel-derived guidelines for the use of brain-dead subjects in medical research have been established, and one institution developed a separate and specialized institutional review committee for this purpose (31, 35). To our knowledge, there are only two reported cases in which a patient has been determined to be brain-dead and subsequently studied as a research subject prior to the removal of life support (36, 37). The present study suggests that the use of brain-dead human subjects in preclinical xenotransplantation studies may be more useful than the current standard of primate experimentation.

Materials and Methods

Cells

All animal experiments were approved by the University of Toledo Institutional Animal Care and Use Committee and completed in the Department of Laboratory Animal Research at the University of Toledo Medical Center, Toledo, OH. Porcine macrophages were obtained via bronchoalveolar lavage (BAL) according to Wensvoort et al. and Brock et al. (38). In short, porcine lungs were carefully excised, and lavaged with approximately 1500 mL (2×750 mL flushes) of cold 0.9% saline (Baxter Healthcare, Deerfield, IL). Lungs were massaged in order to facilitate the release of resident alveolar macrophages. All animals were treated in accordance with the Institute for Laboratory Animal Research (ILAR) publication, *The Guide for the Care and Use of Laboratory Animals* (National Research Council, National Academy Press, Washington, D.C., 1996) and the Animal Welfare Act. Under sterile conditions, the procedure was done quickly in order to preserve cell viability. The BAL fluid was then aliquoted into 50 mL conical tubes and centrifuged at $200 \times g$ for 5

min. The supernatants were decanted and cells were suspended in Roswell Park Memorial Institute Medium (RPMI-1640, Mediatech, Manassas, VA). Cells were then layered onto lymphocyte separation media (Mediatech,) and spun at 500 x g for 40 min, at which time mononuclear cells were extracted from the mononuclear cell monolayer. Cells were then washed in Hanks Balanced Salt Solution (HBSS, Life Technologies, Carlsbad, CA) and re-suspended at a concentration of 10×10^6 in chilled RPMI 1640 and supplemented with 10% fetal bovine serum (FBS, Life Technologies), 1% penicillin/streptomycin (100 U/mL, 100 mg/mL, Life Technologies), and 5% dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO). Samples remained frozen in liquid nitrogen until used. They were removed and placed into culture for 4 days before being utilized in experiments.

Nonhuman primate whole blood samples were obtained from the Toledo Zoo (Toledo, OH), the Southwest National Primate Research Center (San Antonio, TX), or the Yerkes National Primate Research Center (Atlanta, GA) through a protocol approved by the University of Toledo Institutional Animal Care and Use Committee. Type O blood was used for whole human blood used in experiments. Written informed consent was obtained for all human volunteers under a University of Toledo Institutional Review Board approved protocol. Human and nonhuman primate whole blood was collected into a lithium-heparin tube and mixed (Thermo Fisher Scientific, Waltham, MA). The whole blood was washed twice with HBSS. A 0.1% red blood cell solution to be applied to cultured macrophages was prepared with RPMI.

Rosetting Assay

Binding—Determination of porcine macrophage recognition of xenogeneic erythrocytes was performed using an erythrocyte rosetting assay as previously described (15). Data represent three independent experiments performed using human and nonhuman primate erythrocytes from three separate individuals per species, except where noted in figure legend. In brief, after 4 days in culture, macrophages were removed from culture flasks and seeded into a 96-well plate at 3×10^4 cells per well in RPMI culturing medium. After 2 h of incubation, RPMI was replaced and macrophages were incubated with a 0.1% erythrocyte solution. After one hour of erythrocyte/macrophage co-incubation, wells were rinsed with RPMI in order to remove unbound erythrocytes. Wells were then allowed to dry and cells were subsequently fixed with cold methanol.

Quantification—After xenogeneic erythrocyte binding, rosettes were quantified using a tetramethylbenzidine (TMB) reaction in which erythrocyte binding is measured based on the presence of oxygen via oxidation and subsequent color change (39). For the TMB reaction, 100 μ l of solution A and B (R & D systems, Minneapolis, MN) was added to each well and allowed to incubate in the dark for 20 minutes. Following incubation, the reaction was stopped with 2M sulfuric acid and the plate was quantified with a spectrophotometer at a wave length of 450 nm. Data were calculated as percent human erythrocyte binding.

Microscopy—In order to verify quantitative data, rosettes were visualized using bright field microscopy. Porcine macrophages were seeded into a 24-well plate and cultured as previously discussed. After co-incubation, unbound erythrocytes were removed and cells

were fixed with 4% paraformaldehyde and then visualized by bright field microscopy using an Olympus FSX100 microscope (Olympus, Tokyo, Japan).

Statistical Analysis

Significant differences between groups were determined using a student's *t*-test. A P-value of <0.05 was considered to be significant. Data are representative of three individual experiments.

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Abbreviation

CMAH	cytidine monophosphate-N-acetylneuraminic acid hydroxylase
ECLP	Extracorporeal liver perfusion
Gal α-1	3-Gal, galactose α-1,3-galactose
hRBC	human red blood cells
KC	Kupffer cells
Neu5Ac	N-acetylneuraminic acid
Neu5Gc	N-glycolylneuraminic acid
NHP	nonhuman primate
pSn	porcine sialoadhesin

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Figure 1. Effect of CMP-Neu5Ac hydroxylase (CMAH) on macrophage recognition of xenogeneic erythrocytes susceptibility to porcine macrophages. **(A)** Phylogenetic tree showing evolutionary differences of species within the Ape and Old World monkey families. Black arrow indicates time of an *Alu*-mediated disruption of the CMAH gene. **(B)** Structure of the “human form” of sialic acid (Top), Neu5Ac; and the hydroxylated “nonhuman primate form” arising from CMP-Neu5Ac hydroxylase activity. The black arrow represents the addition of the hydroxyl group and the formulation of Neu5Gc resulting from the activity of CMP-Neu5Ac hydroxylase. **(C)** Quantification of porcine macrophage binding to human, chimpanzee, and autologous porcine erythrocytes was determined using a colorimetric rosetting assay. Samples were prepared in triplicate. Data are representative of three independent experiments performed using human positive control erythrocytes from three different individuals. The means and standard errors are shown. Percentage of binding is

presented relative to human erythrocyte binding, which was set at 100%. Significantly more human erythrocytes were bound as compared to chimpanzee erythrocytes ($P<0.001$). **(D)** Representative microscopy images of porcine alveolar macrophages (PAM) illustrate human erythrocyte (top, hRBC) and chimpanzee erythrocyte (bottom, cRBC) binding patterns.

Figure 2.

Porcine macrophage binding to xenogeneic erythrocytes obtained from nonhuman primates most commonly used in xenotransplantation pre-clinical studies. **(A)** The ability of porcine macrophages to recognize erythrocytes from baboons was determined using a quantitative colorimetric erythrocyte rosetting assay. Baboon erythrocytes binding was significantly less than human erythrocyte binding ($P<0.001$). Phase contrast microscopy results further supported the findings obtained with the quantitative rosetting assay. Binding of Cynomolgus monkey **(B)** and Rhesus macaque erythrocytes **(C)** to porcine macrophages was quantified relative to human erythrocyte binding. Binding of erythrocytes from Cynomolgus monkeys and Rhesus macaques was significantly less than human erythrocyte binding ($P<0.001$). Data are representative of three independent experiments performed using human positive control erythrocytes from three different individuals. **(D)** The ability of porcine macrophages to recognize erythrocytes from various nonhuman primates was quantified. Binding of erythrocytes from all five nonhuman primate species tested was significantly less than that of human erythrocyte binding ($P<0.001$). Three independent experiments were done using erythrocytes from three individuals for the human positive control and nonhuman primate erythrocytes from single animals.