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PIG KIDNEY GRAFT SURVIVAL IN A BABOON FOR 136 DAYS: LONGEST LIFE-SUPPORTING ORGAN GRAFT SURVIVAL TO DATE

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Abstract

The longest survival of a nonhuman primate with a life-supporting kidney graft to date has been 90 days, though graft survival >30 days has been unusual. A baboon received a kidney graft from an α 1,3-galactosyltransferase gene-knockout pig transgenic for two human complement- and three human coagulation- regulatory proteins (though only one was expressed in the kidney). Immunosuppressive therapy was with ATG+anti-CD20mAb (induction) and anti-CD40mAb +rapamycin+corticosteroids (maintenance). Anti-TNF- α and anti-IL-6R were administered. The baboon survived 136 days with a generally stable serum creatinine (0.6–1.6mg/dL) until terminally. No features of a consumptive coagulopathy (e.g., thrombocytopenia, decreased fibrinogen) or of a protein-losing nephropathy were observed. There was no evidence of an elicited anti-pig antibody response. Death was from septic shock (*Myroides* spp). Histology of a biopsy on day 103 was normal, but by day 136 the kidney showed features of glomerular enlargement, thrombi, and mesangial expansion. The combination of (i) a graft from a specific genetically-engineered pig, (ii) an effective immunosuppressive regimen, and (iii) anti-inflammatory agents prevented immune injury and a protein-losing nephropathy, and delayed

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Addendum

Since this experiment was concluded, Higginbotham et al (*Xenotransplantation* 2015 doi: 10.1111/xen.12166) have reported GTKO/CD55 pig kidney graft survival in two monkeys for >125 days. They achieved success when they (i) selected monkeys with low anti-pig antibody levels (but not with high antibody levels), and (ii) administered an anti-CD154mAb-based regimen (but not a belatacept-based regimen). It is encouraging that two groups have now extended survival beyond the previous maximum of 90 days.

DISCLOSURE OF CONFLICT OF INTEREST

David Ayares and Carol Phelps are employees of Revivacor, Inc. No other author has a conflict of interest.

coagulation dysfunction. This outcome encourages us that clinical renal xenotransplantation may become a reality.

Keywords

Anti-IL-6R antagonist; Costimulation blockade; Kidney; Pig, genetically engineered; Xenotransplantation

Introduction

Pig organ graft survival in a nonhuman primate (NHP) has been comprehensively reviewed (1, 2). The longest graft survival to date has been 90 days (3), but survival >30 days has been unusual. We here report survival of a baboon with a life-supporting pig kidney for 136 days in which, in contrast to previous reports, features of consumptive coagulopathy and proteinuria were minimal.

Case Report

The kidney was taken from an α 1,3-galactosyltransferase gene-knockout pig transgenic for two human complement-regulatory proteins (CD46, CD55) and three human coagulation-regulatory proteins (thrombomodulin [TBM], endothelial protein C receptor [EPCR], CD39), weight 7.8kg, blood type O (nonA) (Revivicor, Blacksburg, VA) (4). The promoters for the transgenes were CD46 (endogenous), CD55 and EPCR (CAG), and TBM and CD39 (porcine ICAM-2). The expression of CD46, CD55, and EPCR on isolated pig peripheral blood mononuclear cells measured by flow cytometry was high, but expression of TBM and CD39 was low (Figure 1A). By immunofluorescence and immunohistochemistry (carried out retrospectively), no expression of TBM or CD39 could be detected in the kidneys (Figure 1B).

The recipient baboon (weight 8.3kg, blood type B) was from the specific pathogen-free colony at the University of Oklahoma Health Sciences Center (Oklahoma City, OK) (5). Animal care was in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1985). Protocols were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

The immunosuppressive, anti-inflammatory, and adjunctive therapy is detailed in Table 1. Therapy was based partly on our own previous studies (6–8) and partly on those of Mohiuddin and his colleagues (9–11).

Clinical course

The operation was carried out on March 20th, 2014. The surgical procedures have been described previously (12, 13). The kidney functioned immediately.

Post-transplantation, the baboon remained quiet and relatively anorexic for two weeks (for uncertain cause), but then became active and ate and drank well. Urine output was good (approximately 100ml/day), serum creatinine normalized quickly (Figure 2A), and proteinuria and albuminuria (Figure 2B) were generally minimal or modest (little more than we measure in immunosuppressed baboons with pig heart grafts). Serum protein and albumin levels (Figure 2C) remained within the normal ranges. No peripheral edema developed. C-reactive protein, an indicator of inflammation, was minimal as a result of tocilizumab therapy (8).

After 14 weeks, although urine output remained adequate, the serum creatinine slowly increased (Figure 2A). Ultrasound demonstrated an enlarged kidney (Table 2) with good blood flow, and a dilated ureter but with minimal hydronephrosis, suggesting a ureteric stricture at the anastomosis with the bladder.

On day 103, the abdomen was explored, the ureteric stricture was excised, and the ureter re-anastomosed to the bladder. Histological examination of the excised portion of ureter demonstrated no features suggestive of rejection (not shown). The kidney was macroscopically normal except that it had approximately doubled in size (Table 2), and a needle biopsy was taken, which showed no significant histopathological abnormalities (Figure 3A). Immunohistochemical examination revealed no deposition of IgM, minimal deposition of C3, and moderate deposition of IgG (Figure 3B), when compared with control (pre-transplant) sections of naïve pig kidneys.

The serum creatinine fell, but not to the low levels seen initially after the transplant (Figure 2A). On the following day, the baboon experienced what appeared to be a “neurological event” (with stiff limbs, but mentally alert with no paralysis). No obvious cause could be ascertained (the white blood cell [WBC] count was 7,500/ μ l, and all other tests were normal except for a sodium of 153mEq/L). Although the baboon recovered clinically within 24h, it did not eat or drink as well as before the surgical procedure and so a single catheter was placed in a branch of the femoral vein for fluid administration (day 105). Thereafter, despite a lower activity level, the baboon continued to pass good quantities of urine (about 200ml/day).

On day 136, it suffered a similar “neurological event”, again with no obvious cause (WBC count 8,300/ μ l, sodium 153mEq/L).

To ensure adequate hydration and renal blood flow, inotropic support and increased intravenous fluid support was initiated. However, the baboon’s circulatory state deteriorated quickly and it died as euthanasia was being considered. Blood culture was positive for *Myroides spp*, which has not been reported previously in any immunosuppressed patient or NHP with an organ graft (14). *Myroides spp*. are Gram-negative, non-fermentative, obligately aerobic, yellow-pigmented, and non-motile rods, with a characteristic fruity odor. They are a rare cause of bloodstream infection, but have been isolated from several different clinical specimens, and have been implicated in small nosocomial outbreaks. They are characterized by resistance to a wide range of antimicrobial agents that have satisfactory activity against other Gram-negative bacteria (14).

The clinical features suggested that death was associated with septic shock related to the infection and not to kidney graft failure. Necropsy was carried out.

Hematologic parameters

Following induction therapy with ATG and anti-CD20mAb (Table 1), the WBC count remained low ($\pm 1,500/\mu\text{l}$) throughout most of the course of the experiment, but increased into the normal range temporarily after the second operation and terminally. The total lymphocyte count remained consistently at $100\text{--}200/\mu\text{l}$. The red blood cell count, hemoglobin, and hematocrit remained slightly below the normal ranges (for humans) in the absence of any blood transfusion. Importantly, no sustained thrombocytopenia or reduction in plasma fibrinogen levels was observed (Figures 2D and 2E), though there was an immediate post-transplant rise in D-dimer, which, after falling temporarily within one month, then persisted for the remainder of the experiment (Figure 2F).

Immune monitoring

Before transplantation, the baboon had high serum levels of anti-nonGal IgM antibodies (similar to levels seen in baboons previously sensitized to pig antigens) and significantly higher than we have documented previously in baboons from the specific pathogen-free colony (5) (Figure 4A). (It was for this reason only that two doses of cobra venom factor were administered to prevent antibody-mediated complement activation [Table 1].) However, there was no evidence of the development of elicited anti-nonGal IgM or IgG antibodies, indicating no sensitization to nonGal antigens (Figure 4B).

Necropsy and histopathology

The pig kidney graft increased in size throughout the course of the experiment (Table 2), and showed surface and internal features of patchy hemorrhage (Figure 3C and 3D). The ureter remained dilated, but there was no restriction at the anastomosis with the bladder. Low- and high-power histopathological examination of the pig kidney graft at necropsy (day 136) showed widespread focal hemorrhage, features of thrombotic microangiopathy (glomerular enlargement, widespread thrombosis of primarily small vessels in both glomeruli and the interstitium associated with focally extensive infarction and tissue destruction, but little fibrosis, and mesangial expansion); cell infiltrates were minimal (Figure 3E). Immunohistopathology confirmed some C3 and IgG deposition (not shown).

Although these histopathological features may be associated to some extent by the septic state of the baboon, the other major organs showed few changes. We therefore conclude that at least some of these changes were related to an immune response and/or thrombotic microangiopathy and/or inflammation.

Discussion

This experiment had several very encouraging features. (i) Life-supporting survival was 50% longer (136d vs 90d) than in any previously reported pig solid organ xenograft. In contrast to all previous pig kidney xenotransplants (1, 2), (ii) minimal features of consumptive coagulopathy were detected, and (iii) proteinuria was not problematic and not

sufficient to require i.v. replacement with human albumin (3, 15–19). The relative absence of proteinuria suggests that, when previously reported, it was associated with an ongoing immune/inflammatory response, and was not due to an inherent physiologic incompatibility between pig and primate kidneys. (iv) Despite an abnormally high pre-transplant serum anti-nonGal IgM level, the graft did not undergo rapid antibody-mediated injury, and the baboon did not become sensitized to pig antigens.

What factors lead to this relative success? Although further studies will be required to determine the exact role of each, we suggest that important factors were (i) the phenotype of the donor pig, (ii) the costimulation-directed immunosuppressive regimen, and (iii) the anti-inflammatory regimen.

Pig phenotype

It is likely that the absence of Gal expression, and high expression levels of two human complement- and one human coagulation-regulatory protein, EPCR (Figure 1A and B) provided protection from antibody-mediated complement activation and the early development of thrombotic microangiopathy and/or consumptive coagulopathy (7, 8, 10, 11). We suggest that EPCR may have augmented porcine TBM function. A beneficial effect of EPCR alone has also been reported by the University of Maryland group in their perfusion model where pig lungs are perfused with human blood (20). The phenotype of the organ-source pig was probably a major factor in the prolonged survival of the graft.

Costimulation blockade

Based on our own experience (6–8) and that of Mohiuddin and his colleagues (9–11), anti-CD40mAb (specifically 2C10R4) therapy successfully prevents the adaptive immune response to a genetically-engineered pig graft, and is not associated with the thrombogenicity observed with anti-CD154mAb therapy.

The role of induction therapy with anti-CD20mAb is less certain. We have previously reported a high incidence of infectious complications when this agent was administered (7), but we were persuaded of its value by the excellent results reported in the heterotopic heart transplant model (9–11). However, as there has been a trend for reduced doses to be administered to patients receiving ABO-incompatible allografts in recent years, or omitted entirely (Montgomery R, personal communication), we determined to give a single dose of 10mg/kg rather than the full course previously used (20mg/kg×4 at weekly intervals) (9–11). Certainly, the outcome in the present experiment significantly improves on all previous kidney xenotransplantation studies. While it does not match the one and two years survival reported for heterotopic heart transplants, it should be remembered that (i) the kidney was fully life-supporting, and (ii) we administered no intravenous heparin to the baboon after transplantation (although we did administer low molecular weight heparin s.c.), whereas the recipients of the heterotopic heart grafts received a continuous intravenous heparin infusion throughout the course of the experiment (9–11), potentially reducing both thrombosis and inflammation.

Nevertheless, the immunosuppressive regimen may have been excessive in view of the development of a fatal infection in the baboon, and may therefore require modification, possibly by reducing the dosages of some of the agents.

Anti-inflammatory therapy

There is increasing evidence for a prolonged inflammatory response to a pig xenograft (8, 21), and we suggest this is playing a greater role in graft failure than previously believed. We suggest that the isolated rise in D-dimer, which we have observed after both heart and artery patch xenotransplantation (6–8), is an indicator of an inflammatory response rather than of a consumptive coagulopathy. We have data suggesting that the cytokine interleukin-6 (IL-6) is playing a role, and that IL-6R blockade reduces this response (8). We have no personal data that tumor necrosis factor- α (TNF- α) is important, but its perceived benefit in islet allo- and xeno-transplantation persuaded us to incorporate a short course in our protocol. In the present case, we tentatively suggest that the deterioration in graft function after the second operation might have been related to a renewed inflammatory response (induced by the surgical procedure) in addition to the pro-inflammatory effect of infection with *Myroides spp.*

Increase in size of pig kidney

One other observation made during the course of the present study requires comment, and that is the increase in size of the kidney graft that occurred. This was not a result of rejection, when interstitial hemorrhage, thrombosis and cellular infiltrates might increase graft weight. Neither did it appear to be related to severe hydronephrosis associated with the ureteric stricture. Rather, it was a continuing increase in the dimensions of the kidney that was first documented by ultrasound at a time when no functional or histopathological features of rejection were identified (Table 2).

It is possible that, as the demands of the baboon on the single, small kidney may have been greater than those of the young pig, it was associated with a compensatory enlargement of the glomeruli and expansion of the interstitial tissues. Relatively rapid growth in kidney allografts takes place when kidneys from infants or children are transplanted into adult recipients. This may have been a major factor here as it was required to function in the place of two larger baboon kidneys. However, such considerable growth does not appear to have been reported previously after xenotransplantation.

Conventional opinion has been that the transplanted pig organ is sensitive to primate growth hormone, and therefore the organ would grow in size in concert with the primate's own organs (15, 16). This was the case in the long-surviving pig heart grafts in baboons reported by Mohiuddin (11). Furthermore, an enlarged (CD55-transgenic) kidney phenotype was not reported after 90 days survival (17). Nevertheless, the pig kidney could have grown at the rate it would have done if in its natural environment (in the pig). Thus, further investigation of this observation is required.

This kidney transplant was the first and only one to date that we have carried out with this specific genetically-engineered pig and immunosuppressive regimen. Indeed, it was the only

pig kidney transplant we have carried out in several years, as we have concentrated our efforts on heart transplantation. Since completing this single experiment, we have carried out two more kidney transplants using an identical regimen but a different genetically-engineered pig donor. Both grafts unfortunately failed within a few days after the transplant, which evidence suggests was from ischemic injury associated with inadvertent inadequate cooling and preservation of the kidneys.

In summary, we are encouraged by the prolonged survival of this baboon with a life-supporting pig kidney graft. We suggest that the pig phenotype, costimulation blockade-based immunosuppressive regimen, and anti-inflammatory therapy may all have contributed to the outcome.

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ABBREVIATIONS

EPCR	endothelial protein C receptor
GTKO	galactose- α 1,3-galactose (Gal) gene-knockout
IL-6R	interleukin-6 receptor
NHP	nonhuman primate
TBM	thrombomodulin

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A **B** **Figure 1.**

(A) Expression of human transgenes on donor pig PBMCs by flow cytometry. Gal expression was absent, expression of CD46, CD55, and EPCR was high, whereas expression of TBM and CD39 was low.

(B) Expression of human transgenes on donor kidney by immunofluorescence or immunohistochemistry. (Left to right) CD46, CD55, TBM (not detectable), EPCR, CD39 (not detectable). Studies on CD46, CD55, and EPCR were by immunofluorescence but, in view of a high background activity, immunohistochemistry was used for TBM and CD39. This may possibly influence the results. Although there appears to be a discrepancy between expression of CD55 and EPCR, their expression should be equimolar as a single transcript is produced to join these genes. The difference in intensity in these images may be related to specific antibody affinity rather than significant differences in expression.

A

B

C

D

E

F **Figure 2.**

Monitoring of recipient baboon throughout the course of the experiment. (A) serum creatinine; (B) urinary protein; (C) serum albumin; (D) platelet counts; (E) fibrinogen; (F) D-dimer. The horizontal lines indicate either the upper and lower limits (A, C, D) or lower limit (E), or upper limit (F), of the equivalent parameters measured in healthy humans.

A

B

C

D

Figure 3.

(A) Microscopic appearance of the pig kidney graft on biopsy on day 103 (magnification x20). Although there has been some artifact distortion of the tissue in the preparation of the section, the histology is basically normal.

(B) Immunohistochemistry staining of the pig kidney graft on biopsy on day 103, demonstrating minimal C3 (left), moderate IgG (center), but no IgM (right) deposition, suggesting an elicited antibody response.

(C) Macroscopic appearance of the pig kidney graft at necropsy on day 136.

(D) Macroscopic appearance of a cross-section of the kidney graft at necropsy on day 136.

(E) Low-power (left, magnification x4) and high-power (right, x40) microscopic appearance of the pig kidney graft at necropsy on day 136 from an area with macroscopic hemorrhage.

The sections show widespread focal hemorrhage and features of thrombotic microangiopathy (thrombosis of primarily small vessels with focally extensive infarction and tissue destruction); cell infiltrates were minimal. The thrombotic microangiopathy is characterized in part by multiple occluding thrombi within glomerular capillary loops and associated general mesangial expansion.

Figure 4.

(A) Pre-transplant anti-pig nonGal IgM and IgG levels (determined by flow cytometry) in the recipient baboon (open circle and square) and in 9 other baboons (closed circles and squares) from the Division of Animal Resources, Oklahoma University Health Sciences Center, specific pathogen-free colony received in Pittsburgh during the past 2 years.

(B) Anti-pig nonGal IgM (left) and IgG (right) levels in the recipient baboon pre-transplantation (solid line), 60 days post-transplant (dotted line), and 136 days post-transplant (dashed line). (Isotype control - shaded).

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Table 1

Immunosuppressive, anti-inflammatory, and adjunctive therapy

Agent	Dose (Duration)
<u>Immunosuppressive</u>	
<u>Induction:</u>	
Thymoglobulin (ATG) (Genzyme, Cambridge, MA)	10mg/kg (day -3)
Anti-CD20mAb (Rituximab) (Genentech, South San Francisco, CA)	10mg/kg (day -2)
Cobra venom factor (Complement Technology, Tyler, Texas)	100IU (days-1 and 0)
<u>Maintenance:</u>	
Anti-CD40mAb (2C10R4) (NIH NHP Resource Center, Boston, MA)	50mg/kg (days -1, 0, 4, 7, 14, and weekly)
Rapamycin (LC Laboratories, Woburn, MA)	0.01mg/kgx2/day (target 8–12ng/ml) (from day -3)
Methylprednisolone (MP) (Astellas, Deerfield, IL)	5mg/kg/day tapering to 0.25mg/kg/day
<u>Anti-inflammatory</u>	
Tocilizumab (IL-6R blockade) (Genentech, South San Francisco, CA)	10mg/kg (days -1, 7, 14 and every 2 weeks)
Etanercept (TNF- α antagonist) (Amgen, Thousand Oaks, CA)	0.5mg/kg (days 0, 3, 7, 28, 40)
<u>Adjunctive:</u>	
Aspirin (Bayer, Deland, FL)	40mg p.o. (alternate days)
Low molecular weight heparin (LMWH) (Eisai, Woodcliff Lake, NJ)	700 IU/day s.c

Table 2

Approximate dimensions of pig kidney determined by direct measurement or ultrasound

Time	Dimensions (in cms) (length × width at hilum × thickness)
Day 0 (day of kidney transplantation)	5 × 3 × 3
Day 82 (ultrasound)	9 × 5 × 3
Day 99 (ultrasound)	11 × 6 × 4
Day 103 (day of reoperation)	14 × 7 × 4
Day 136 (necropsy)	15 × 9 × 6