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Results of Life-Supporting GalT-KO kidneys in Cynomolgus Monkeys Using Two Different Sources of GalT-KO Swine

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Abstract

Background—Various durations of survival have been observed in the xenotransplantation of life-supporting alpha-1,3-galactosyltransferase knockout (GalT-KO) porcine kidneys into nonhuman primates (NHPs). While others have demonstrated loss of GalT-KO transplanted kidneys within two weeks, we have reported an average survival of 51 days with the co-transplantation of the kidney and vascularized thymus and an average of 29 days with the kidney alone. In order to determine the factors responsible for this difference in survival time, we performed xenogeneic kidney transplantations into cynomolgus monkeys with an anti-CD40L-based regimen using two different strains of GalT-KO swine, one derived from MGH-Miniature swine and the other obtained from Meiji University.

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Authorship contribution

Mitsuhiro Sekijima primarily performed the in vivo experimental procedures, analyzed data, and participated to write this manuscript.

Shiori Waki primarily performed the in vitro assays, analyzed data, and participated to write this manuscript.

Hisashi Sahara supervised in vivo experimental procedures and analyzed data.

Masayuki Tasaki participated performed the in vivo experimental procedures and analyzed data.

Vincenzo Villani participated in analyzing data.

Yoshiki Shimatsu primarily produced GalT-knockout MGH miniature swine using NIBS swine surrogates.

Kazuaki Nakano, Hitomi Matsunari, and Hiroshi Nagashima primarily produced GalT-knockout MEIJI swine

Jay Fishman and Robert Wilkinson designed the molecular assays for viral infection.

Akira Shimizu examined the histopathology.

Kazuhiko Yamada primarily designed the experimental protocol, performed the experimental procedures, analyzed data, and wrote this manuscript, and is the corresponding author.

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Materials and Methods—Eight cynomolgus monkeys received GalT-KO kidneys. Three kidney grafts were from MGH/NIBS GalT-KO pigs and 5 GalT-KO grafts were from MEIJI GalT-KO swine. All cynomolgus recipients were treated identically.

Results—Recipients of kidneys from the MGH GalT-KO swine, produced by nuclear transfer in Japan, survived an average of 28.7 days, while recipients of MEIJI GalT-KO swine survived an average of 9.2 days. Among the differences between these two groups, one potentially revealing disparity was that the MEIJI swine were positive for porcine-CMV, while the MGH-derived swine were negative.

Conclusions—This is the first study comparing renal xenotransplantation from two different sources of GalT-KO swine into NHPs at a single center. The results demonstrate that porcine-CMV may be responsible for early loss of GalTKO swine kidney xenografts.

Keywords

GalT-KO swine; Xenotransplantation; Life-supporting kidney xenografts; Porcine CMV

INTRODUCTION

Although improvements in operative technique and immunosuppression have paved the way for increases in the organ donor pool through living donor kidney donation, there remains a vast disparity between the number of organs available for transplantation and the demand for these organs. Because of favorable breeding characteristics as well as physiological and anatomical similarities to humans, pigs are viewed as the most likely source of organs for future xenotransplantation (1). Until recently, hyperacute rejection (HAR), caused by preformed natural xenoreactive antibodies directed against the sugar alpha-galactose-1,3-galactose (Gal) (2), was a major hurdle in pig to human discordant xenotransplantation. To address this problem, in 2002 two groups produced knockout pigs (GalT-KO) which do not express the Gal epitope (3,4). Since the first successful production of GalT-KO pigs (3,4), additional genes have been manipulated in GalT-KO pigs (5-7).

In collaboration with our colleagues at the Transplantation Biology Research Center (TBRC), we have reported that the elimination of Gal antigens avoided hyperacute xenograft rejection such that life-supporting GalT-KO kidney grafts in baboons had an average survival of 29 days (8). Moreover, GalT-KO kidney function was markedly prolonged when a vascularized thymus co-transplantation was performed in baboon recipients of MGH GalT-KO kidneys, with survivals without rejection averaging 51 days and with some survivals up to 83 days (8,9). However, the graft survival of GalT-KO kidneys in non-human primates as reported by other groups were limited and most grafts were lost within 16 days following transplantation (10,11).

In an attempt to determine the cause of this difference in life-supporting kidney xenograft survival time between the TBRC experience and that of other groups, we have performed xenogeneic kidney transplants without co-transplantation of thymus using two different sources of GalT-KO swine (12,13).

RESULTS

Recipients of MGH/NIBS kidneys maintained graft function up to day 30 (Group 1) while all recipients of MEIJI GalT-KO kidneys developed either severe rejection or disseminated intravascular coagulation (DIC) by day 15 (Group 2)

Recipients of MGH/NIBS kidneys

Three recipients of MGH/NIBS kidneys, shown in Figure 1A, maintained stable creatinine levels in the first two weeks. S-Cre in one of three recipients increased from post-operative day (POD) 17 and reached at 6.61 mg/dl at POD 27. The others maintained xenograft function for 4 weeks; one eventually lost function at POD 30 while the other maintained S-Cre of less than 1.6 mg/dl up to POD 29 when the animal was sacrificed due to respiratory complications secondary to proteinuria. The recipients of MGH/NIBS GalT-KO kidneys maintained kidney function for an average of >28.7 days with one that had normal S-Cre at POD 29. The hematologic parameters followed a similar pattern in these recipients. Although the platelet (PLT) levels decreased from POD17 in the one that lost its xenograft at POD 27, the other two had stable PLT levels (Fig 1B).

Notably, pathological examination of the excised kidney from the 30 days survivor (M23408) had no indications of rejection (Fig 2A). Histologic findings of GalT-KO kidneys in others two recipients (M23411 and M23413) showed interstitial mononuclear cell infiltrates with tubulitis (Fig 2B) which were similarly seen in previous report of GalT-KO kidneys without tolerance strategy (13).

Recipients of MEIJI kidneys

In contrast, S-Cre levels increased as early as POD 7 in five recipients of MEIJI GalT-KO kidneys (Fig 1C) and all recipients died or were euthanized subsequent to either complete rejection or DIC like symptoms within 15 days. The recipients of MEIJI GalT-KO kidneys survived an average of 9.2 days. The two animals that died one or two days after their S-Cre began to increase (1.54mg/dl and 2.39mg/dl, respectively; Fig 1C). These animals died from pleural effusion with either severe thrombocytopenia (PLT/Htc 35K/ul/24.2%) or severe anemia (PLT/Htc 290K/ul/15.5%) at the time of death (Fig 1D).

The graft histology in the longest surviving recipient at POD 15 (M22405) showed interstitial hemorrhages with microthrombi in glomeruli (Fig 2C), glomerular microangiopathy with thrombus in arteriole (Fig 2D), and endothelial activation with microthrombi in small artery (Fig 2E. More details in later section).

Assessment of parameters in Group 1 and Group 2

Preformed non-Gal Nab and induced anti-porcine antibodies in sera—The presence of serum non-Gal Nab prior xenogeneic kidney transplantation (preformed non-Gal Nab) and at the time of necropsy in the recipients was determined by FACS analysis. Average of median fluorescence intensity (MFI) was not significantly different in the recipients of both MGH/NIBS and MEIJI kidneys (MFI at pre-transplant: 67 ± 8 versus

72±13; time of necropsy: 45±3 versus 44±5, Fig 3). No induced anti-pig antibodies developed in either recipients of MGH/NIBS or MEIJI kidneys.

Source of monkeys—All monkeys in this study were provided by the Shin Nippon Biomedical Laboratories, Ltd., Kagoshima, Japan and they were housed in a closed environment and randomly enrolled in either group.

Housing environment of donors—There are major differences in the breeding environments between the MGH/NIBS GalT-KO and the MEIJI GalT-KO pigs. MGH/NIBS GalT-KO swine were bred and housed in an experimental facility at the NIBS research institute in Yamanashi, Japan. All swine in the facility are routinely screened for several viruses (Japanese Encephalitis virus, Getah virus, Porcine Parvovirus, Transmissible Gastroenteritis virus, Porcine Epidemic diarrhea virus, Aujeszky's disease virus, Swine fever virus, Porcine Reproductive and Respiratory virus syndrome virus); bacteria (*Actinobacillus pleuropneumoniae* (Serotypes: 1, 2, 5), *Haemophilus parasuis* (Serotypes: 2, 5), *Bordetella bronchiseptica*, *Pasteurella multocida* (toxin producing), *Erysipelothrix rhusio-pathiae*, *Mycoplasma hyopneumoniae*, *Salmonella* spp); ecto- and endoparasites (*Toxoplasma gondii*, *Metastrongylus elomgatus*, *Ascaris suum*, *Balantidium coli*) and must remain free of these pathogens in order to minimize potential zoonotic infection post-transplant. In contrast, MEIJI GalT-KO swine surrogates and piglets were housed in a commercial farm in Ibaragi, Japan, until they were shipped to the Kagoshima University. All pigs shipped from each facility were housed in the Animal facility at Kagoshima University in isolated cages to minimize pig to pig contact.

Surgical procedure and ischemic time—All of the transplants, donor harvests and anastomosis of renal vessels and ureters were performed by the same surgical team (lead by K. Yamada). The donor surgery and recipient surgery were performed in the same operating room and a total anastomosis time for both renal artery and vein was an average of 22±2 min.

pCMV—The pCMV status of the donors and the transplanted kidneys were assessed by PCR. All of the DNA from the MGH/NIBS pig kidneys were negative for pCMV even after xeno kidney transplants with immunosuppression (Fig 4A left). In contrast, the pCMV PCR product was amplified in all of the MEIJI GalT-KO samples both pre- and post-xenotransplantation (Fig 4A right).

Cynomolgus CMV (CyCMV)—In order to assess the presence of CyCMV in the recipients as well as to rule out the possible cross-reactivity of primers, recipients of MEIJI GalT-KO kidneys were tested for the presence of the virus at the time of necropsy by PCR. As shown Fig 4B, CyCMV was not detected in any of the MEIJI GalT-KO kidneys (Fig 4B left). In addition, tissues tested (lung, livers and lymph nodes) from all recipients were CyCMV negative (Fig 4B right).

Immuno-pathological assessment of endothelial cell activation and the presence of pCMV

Although pCMV was positive in PCR in MEIJI GalT-KO kidney grafts, no inclusion body was found in tested samples (data not shown). However, MEIJI GalT-KO kidneys had histologic evidences of more profound activation of endothelial cells than those in MGH/NIBS GalT-KO kidneys despite of similar levels of S-Cre. Fig 5 showed PAS, factor VIII, vimentin and PCNA staining of a MEIJI kidney that died at day 6 with S-Cre 1.54 mg/dl and an MGH/NIBS kidney at day 29 with S-Cre 1.59 mg/dl.

PAS staining showed that peritubular capillaries of pCMV negative kidney well-opened with small endothelial cells (Fig 5A) while pCMV positive MEIJI kidney showed peritubular capillaries parted from tubules and their lumen was narrow with swollen endothelial cells (Fig 5E). Expressions of factor VIII that is a von Willebrand factor produced by endothelial cells and vimentin, a type III intermediate filament protein at endothelial cells in peritubular capillaries are upregulated in the pCMV positive MEIJI kidney graft (Fig 5 F, G) but only minimal positive cells were seen in the pCMV negative MGH/NIBS graft (Fig 5 B, C). In addition, obvious numbers of PCNA positive cells were seen in pCMV positive MEIJI grafts (Fig 5H) but there is only few in the pCMV negative MGH/NIBS graft (Fig 5D).

DISCUSSION

Our present report was designed as an attempt to determine the cause of the difference in life-supporting kidney xenograft survival times as reported by the TBRC (average 29 days with stable renal function in the first 2 weeks) (13) and other groups (unstable renal function and most of kidneys rejected within 16 days) (15,22,23). For this purpose, we performed xenogeneic kidney transplants, without co-transplantation of thymus, using two different sources of GalT-KO swine. The results in this report demonstrate that: (i) the recipients of MGH/NIBS GalT-KO kidneys maintained kidney function for an average of >28.7 days which was similar to previous reports from the TBRC; (ii) the recipients of MEIJI GalT-KO kidneys survived an average of 9.2 days, similar to the results from other research institutes (15,22,23); (iii) no MGH/NIBS GalT-KO donor kidneys were pCMV positive while all of the MEIJI GalT-KO kidneys were positive even prior to transplantation, suggesting pCMV may be the causative agent of early porcine kidney rejection in non-human primates (NHP) recipients of GalT-KO pig kidneys.

In order to minimize variation in procedures, all of the transplants in this study were performed at Kagoshima University by the same surgical team led by Yamada who led GalT-KO kidney experiments at TBRC and all recipients received the same immunosuppressive regimen. Vascular anastomoses of both arteries and veins were completed in an average of 22 min and no surgical acute tubular necrosis was induced. These conditions resulted in stable renal function in the first 3 days following transplantation. All other parameters, source of recipient cynomolgus monkeys, preformed non-Gal Nab levels and immunosuppressive regimens, were essentially identical so that there were no obvious differences between the two groups. Only a major difference in the recipients of MGH/NIBS kidneys and recipients of MEIJI kidneys is source of GalTKO pigs and the breeding environments between the MGH/NIBS GalT-KO (17) and the MEIJI GalT-

KO pigs (13). The MGH/NIBS GalT-KO swine were bred and housed in a closed experimental facility at the NIBS research institute that has routine virus screenings while the MEIJI GalT-KO swine surrogates and piglets were housed in a commercial farm that potentially introduces pCMV into the herd. It is important to note that the MGH/NIBS GalT-KO miniature swine that we have used in this study were re-cloned in the NIBS viral free facility from fetal fibroblasts of MGH GalT-KO (8, 13) frozen at the time of the previously reported studies that demonstrated the 29-day survivals (13). This re-cloning as a source of MGH/NIBS GalT-KO is relevant because recent studies at the TBRC have shown that the recent appearance of pCMV in the MGH herd was associated with a markedly decreased graft survival (Yamada K et al. manuscript in press). These studies, performed simultaneously at two research centers using different approaches, uniquely complement each other and provide further confirmation of the potential role of donor PCMV infection in xenotransplant rejection.

We did not detect inclusion bodies histologically in the kidney grafts to demonstrate direct evidence of pCMV infection in the graft. Intracellular inclusions due to PCMV have been identified in prior studies in infected xenografts (14). These are thought to represent primarily viral capsid protein aggregates. However, the sensitivity for detection of CMV inclusion bodies is low and clinically, CMV-specific inclusion bodies can be absent in the transplanted tissues even when CMV DNA and proteins are detected in the graft by PCR and immunohistochemistry (15). This has been attributed, in part, to the effect of antiviral prophylaxis on the normal replication of CMV. We have previously demonstrated that ganciclovir has some activity against porcine CMV in vitro, but it is more effective in treating human CMV (16). The mechanism for the relative resistance to ganciclovir is unknown, but in the presence of the drug viral protein synthesis may be partially disrupted resulting in impaired formation of inclusion bodies. Endothelial activation would be expected during infection even in the absence of inclusions (17), consistent with the immunohistochemistry results in this study which demonstrated that the presence of inclusion bodies may also be altered by the use of antiviral prophylaxis. Our immunohistochemistry results, however, demonstrated marked endothelial cell activation, as determined by Factor VIII, vimentin and PCNA staining in the MEIJI kidneys even in a recipient with S-Cre level 1.54 mg/dl (Fig. 5). In other reports in allogeneic human kidneys as well as rodent models, up-regulation of the expression of ICAM-1 and MHC antigens by CMV associated with allograft rejection has been demonstrated (18-20). Also CMV possibly up-regulates MHC class II expression in kidney allografts via IFN γ released by activated T cells (21). Taken together, these data suggest that intra-graft pCMV which was found in all of MEIJI kidneys at the time of donation, underwent increased replication following transplantation with immunosuppression. This increased replication presumably induced the early activation of endothelial cells and further inflammatory cascades in the porcine kidneys that resulted in the destruction of endothelial cells.

In order to avoid pCMV associated graft loss, two major strategies can be considered: one is pharmacologic treatment and the other is a pCMV-free environment for breeding and housing of donors. Ganciclovir, an antiviral medication, has been widely used to treat symptomatic congenital CMV infection in immunocompromised hosts. We also included Ganciclovir in our immunosuppressive protocol. However, it appears not be as effective on

porcine CMV as it is on primate CMV. Therefore, pCMV specific anti-virus drugs would be desirable. In the absence of such drugs, a pCMV free environment is required for breeding and housing of donors. The NIBS facility for MGH GalT-KO is a porcine specific experimental facility that is well-controlled to minimize virus transmission from outside. However, if a sow is positive, C-section may avoid transmission of pCMV to the offspring. Prevention of maternal and congenital CMV infection may be the most effective way to eliminate post-transplant pCMV infection in NHP recipients of GalT-KO grafts (22,23). Although all of the MEIJI GalT-KO kidneys had detectable pCMV even before they were transplanted into immunosuppressed recipients, latent pCMV could re-activate following transplantation.

This is the first study that has compared the xenotransplantation of two different donor sources of GalT-KO swine kidneys into NHPs in a parallel manner at a single center. Our results demonstrate a clear difference in outcome between MGH/NIBS GalT-KO and MEIJI GalT-KO donor kidneys, likely due to the presence or absence of pCMV in the donor tissue, and suggesting that this virus may be the causative agent of early kidney rejection in NHP recipients of GalT-KO pig kidneys. Our data strongly indicate that a clean environment and careful monitoring reduces the likelihood of transmission of pCMV, or any similar virus, and that such practices could be crucial for successful xeno kidney transplants.

MATERIALS AND METHODS

Animals and experimental groups

All animals were cared for according to the guidelines of the Kagoshima University Institutional Animal Care and Use Committee.

Donor pigs—Two sources of GalT-KO pigs were used in this study. Two MGH/NIBS GalT-KO swine were used as donors of three kidney grafts, and Four MEIJI GalT-KO swine were used as donors of five kidney grafts. Production of MGH/NIBS GalT-KO pigs and MEIJI GalT-KO pigs has been previously reported (12,13), and details are described in Supplemental Digital Content.

Recipients—Male or female recipient cynomolgus monkeys (n=8) were purchased from the Shin Nippon Biomedical Laboratories, Ltd., Kagoshima, Japan.

Experimental groups—Recipients of MGH/NIBS GalT-KO kidneys were enrolled in Group 1 and recipients of MEIJI GalT-KO kidneys were enrolled in Group 2.

Life-supporting kidney transplantation and Immunosuppression

All cynomolgus recipients (n=8) were treated identically. Recipients received splenectomy, nephrectomy of both native kidneys followed by orthotopic kidney transplantation as previously reported (9). GalT-KO kidneys were harvested from donors in the same room as recipient surgery and anastomosis time for both renal artery and renal veins were performed within 30 min. All recipients received the same immunosuppressive regimen including anti-CD40L mAb. Details of Supplemental Digital Content.

Non-Gal Natural antibodies (non-Gal Nab)

The presence of serum non-Gal Nab in the recipients was determined by FACS both pre-transplant and at the time of necropsy. Briefly, heat inactivated sera was incubated with freshly isolated GalT-KO PMBC. Binding of serum Ab was detected with anti-human IgM FITC. (Dako, Denmark). The cells were analyzed with a Becton Dickinson FACSVerse microfluorimeter.

Assessment of kidney xenografts

S-Cre and CBC were monitored daily to assess life-supporting renal function as well as hematologic parameters. Renal xenografts were examined histologically at the time of necropsy. (See details in Supplemental Digital Content)

Cytomegalovirus (CMV)

Sampling of tissue and DNA preparation—Donor tissue samples were collected at the time of nephrectomy while recipient samples were taken at necropsy. DNA was isolated using the DNeasy Blood&Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer protocol.

PCR Assay (porcine CMV and cynomolgus CMV)—Details of CMV assays are described in Supplemental Digital Content.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

GalT-KO	α -1,3-galactosyltransferase knockout
NHPs	nonhuman primates

MGH	Massachusetts General Hospital
pCMV	porcine cytomegalovirus
CyCMV	Cynomolgus CMV
HAR	hyperacute rejection
SCNT	somatic cell nuclear transfer
NIBS	Nippon Institute for Biological Science
Nab	Natural antibodies
S-Cre	Serum creatinine
CBC	complete blood counts
H&E	hematoxylin and eosin
PAS	periodic acid-Schiff
PCNA	proliferating cell nuclear antigen
Cytb	cytochrome b
DIC	disseminated intravascular coagulation
POD	post-operative day
PLT	platelet
MFI	median fluorescence intensity
ConA	concanavalin A

Figure 1.

S-Cre and PLT counts following GalT-KO kidneys in cynomolgus recipients. A, S-Cre of recipients of MGH-NIBS donors, (B) PLT counts of recipients of MGH-NIBS donors, (C) S-Cre of recipients of MEIJI donors, (B) PLT counts of recipients of MEIJI donors. S-Cre, serum creatinine; PLT, platelet; GalT-KO, galactosyltransferase knockout.

Figure 2.

Histologic analysis of the excised kidneys. (A) Histology of an MGH/NIBS kidney from a 29 days survivor (M23408) showed no indications of rejection ($\times 100$ PAM). (B) Interstitial infiltrates (arrow) and tubulitis (arrowhead) are seen in an MGH/NIBS kidney in M2341 ($\times 600$ PAS). (C, D) Histology in the longest surviving MEIJI recipient at POD 15 (M22405) showed focal interstitial hemorrhages with microthrombi in glomeruli (C. $\times 400$ HE), glomerular microangiopathy with thrombus (arrow) in arteriole (D. $\times 600$ PAM), and endothelial activation with microthrombus (arrow) in small artery (E. $\times 600$ PAM).

Figure 3. Serum non-Gal natural antibodies (non-Gal Nab) assessed by FACS. Average of median fluorescence intensity of the five recipients of MEIJI GalT-KO kidneys was similar to that of the three recipients of NIBS GalT-KO kidneys both pre- transplant and at the time of necropsy. As a negative control, medium without serum was incubated with GalT-KO PBMC and then anti-human IgM FITC was added.

Figure 4.

(A) Detection of porcine CMV (pCMV) by PCR. A PCR product of 215 bp was amplified from pCMV. Cytb was included as a control. NIBS/MGH GalT-KO tissues were all negative even after xeno kidney transplants (A left: Lane 1-5). MEIJI GalT-KO tissues were all positive both pre- and post-xenotransplantation (A right: Lane a-i). (B) Detection of cynomolgus CMV (CyCMV) by PCR in the excised tissues from the recipients of MEIJI GalT-KO kidneys at the time of necropsy (B left: graft kidney; B right: lung, liver, lymph nodes). All samples were negative for CyCMV. Lane M: 100 bp DNA ladder (Takara Bio Inc., Ohtsu, Japan). Lane NC: Negative control.

Figure 5. Immuno-pathological examination of endothelial cell activation in excised GalT-KO kidneys in cynomolgous recipients. Samples A, B, C and D were taken from a MGH/NIBS kidney in a recipient at POD 29 (S-Cre 1.59 mg/dl). Samples E, F, G and H were taken from a MEIJI kidney in a recipient at POD 6 (S-Cre 1.54 mg/dl). PAS (A and E), factor VIII (B and F), vimentin (C and G) and PCNA (D and H) staining ($\times 600$ magnification).