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Prolonged Survival of Pig Skin on Baboons following Administration of Pig Cells Expressing Human CD47

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Abstract

Background—Successful xenotransplantation will likely depend, in part, on the induction of immunological tolerance, since the high levels of immunosuppression otherwise required would likely have unacceptable side effects. Rapid clearance of administered porcine hematopoietic stem cells by primate macrophages has hampered previous attempts to induce tolerance through mixed hematopoietic chimerism across a pig-to-primate barrier. Phagocytosis is normally inhibited by binding of cell surface protein CD47 to macrophage SIRPα receptors. However pig CD47 has previously been shown to be ineffective in transducing signals through primate SIRPα.

Methods—Mobilized peripheral blood hematopoietic cells from transgenic swine expressing high or low levels of human CD47 were infused into conditioned baboons at 3 time points over a 9-week period. Xenogeneic peripheral blood chimerism was assessed following each infusion. Split thickness skin grafts from the hematopoietic cell donor swine were placed on recipients 5 weeks following the last cell infusion and 7 weeks following the discontinuation of all immunosuppression to test immune response.

Results—The level and duration of transient chimerism were substantially greater in baboons receiving hematopoietic cells from a pig expressing high levels of human CD47. Skin graft survival on high CD47 recipients was prolonged as well, in one case showing no signs of rejection at least 53 days following placement.

Conclusions—Prolongation of transient porcine chimerism via transgenic expression of human CD47 in a primate model is associated with an immune modulating effect, leading to markedly

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Authorship

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Disclosures

The authors declare no conflicts of interest.

prolonged survival of donor swine skin xenografts that may be applicable to clinical solid organ xenotransplantation.

Introduction

Xenotransplantation of pig organs offers the best near-term solution for relieving the current shortage of transplantable allogeneic solid organs. However, the acquired immune response is considerably stronger to xenografts than it is to allografts, at the levels of both antibody and T cell immune responses^{1–4}, suggesting that clinically relevant levels of conventional immunosuppression will be inadequate to prevent acquired immune rejection of solid organ xenografts.

Both stable and transient lymphohematopoietic chimerism have been demonstrated to be capable of inducing tolerance of solid organ transplants. Thus, studies in murine⁵ and porcine⁶ animal models and in recent clinical trials⁷ have demonstrated that the establishment of *stable* allogeneic mixed lymphohematopoietic chimerism leads to transplantation tolerance of donor organs across MHC barriers. In nonhuman primate models^{8–10} and HLA-mismatched clinical trials^{11;12}, tolerance of allogeneic solid organ grafts has also been achieved in conjunction with establishment of *transient* mixed lymphohematopoietic chimerism.

Extension of these modalities to xenografts has been limited to date by the rapid macrophage-mediated clearance of porcine hematopoietic cells (HC) from the primate circulation, precluding the establishment of either stable or transient chimerism of substantial duration and degree^{13–15}. A likely reason for this clearance is that inhibition of phagocytosis through macrophage SIRP α receptor binding of CD47 on HC does not occur in the pig/primate combination^{16;17}. Consistent with this hypothesis, we have previously demonstrated that transgenic expression of human CD47 (hCD47) substantially increases HC engraftment in a murine model of pig-to-human cell transplantation¹⁸.

In this study, we report that transgenic expression of hCD47 on HC of α -1,3-galactosyltransferase knockout pigs substantially increases the duration of transient xenogeneic chimerism following HC transplantation to baboons. Additionally, prolonged transient chimerism in this model is associated with acceptance of porcine skin grafts for up to 2 months in the absence of concurrent immunosuppression.

Materials and Methods

Animals

Baboon recipients were male *Papio Hamadrayas* (n=5) of 5–6 kg and 2–3 years of age (Mannheimer Foundation, Homestead, FL, USA). All recipients were selected for low pre-transplant anti-non-Gal cytotoxic antibody levels. The derivation of cloned hCD47 transgenic pig 18286 was previously described¹⁸; transgenic pig 18289 was derived in an identical fashion and the genotype confirmed also as described. All animal work was conducted in accordance with NIH and USDA guidelines and with approval from the MGH Institutional Animal Care and Use Committee.

FACS Analysis of hCD47

Cell surface expression of hCD47 was detected using PE or AlexaFluor 647 conjugated mouse-anti-human CD47 clone B6H12 (BD Biosciences) on heparinized whole blood or PBMC. Acquisition was performed on a FACSCalibur (Becton-Dickinson, Mountain View, CA, USA) and analyzed using FloJo software (Treestar, Ashland OR).

Hematopoietic progenitor cell isolation and transplantation

Peripheral blood stem and progenitor cells from hCD47 transgenic swine were obtained at 9–11 months of age by leukapheresis following mobilization for 6 days with porcine IL-3 and porcine SCF as previously described¹⁹. Low density cells from the leukapheresis product were isolated by gradient centrifugation over Histopaque (Sigma, St. Louis, MO, USA), diluted to $p=1.070$. Low density cells were enriched for the ckit+ population using biotinylated porcine SCF as previously described²⁰. The degree of enrichment was assessed by flow cytometry using biotinylated SCF and streptavidin conjugated AlexaFluor 647. High density cells (lymphocytes) were also isolated by gradient centrifugation over Histopaque (Sigma, St. Louis, MO, USA) diluted to $p=1.077$.

Baboons were conditioned with a nonmyeloablative regimen. The regimen consisted of T cell depletion with 250 cGy Total Body Irradiation (TBI), 700 cGy of Thymic Irradiation, 2 doses of a monoclonal anti-CD2 antibody (loCD2b), and 1 dose of 50 mg/kg equine anti-thymocyte globulin (ATGam, Pfizer). B cell depletion was achieved with 1 dose of anti-CD20 (Rituxan, Genentech) 20 mg/kg 9 days prior to cell infusion on Day 0.

Animals received a continuous infusion of tacrolimus maintenance therapy from day 3 to day 28, through indwelling intra-venous catheters, after which the immunosuppression was discontinued and the intravenous catheters removed. The dose of tacrolimus was 0.1 mg/kg/day to achieve a target blood level of 5–10 ng/ml. Anti-CD154 (CD40L) costimulatory blockade was administered at 20 mg/kg on days –1, 1, 3, and 5, then weekly through 7 weeks. Methylprednisolone (Solu-Medrol) at 4mg/kg was given BID from day 0–2. Splenectomy was performed on day –10, at the time of catheter placement. All baboons received prostacyclin and heparin to prevent thrombotic microangiopathy, cytomegalovirus prophylaxis with Ganciclovir and prophylactic levofloxacin and cefepime.

Isolated hCD47 high or low expressing porcine progenitor cells (ckit+) and lymphocytes from the leukapheresis product were intravenously infused into Group 1 and 2 baboon recipients, respectively, on day 0, with additional infusions 7 and 9 weeks later. The second and the third cell infusions consisted of unfractionated total leukapheresed porcine cells that were frozen at the time of collection from the transgenic donors. The control baboon received only the conditioning and maintenance regimen.

Assessment of peripheral blood chimerism

Pig cells were assessed in recipient peripheral blood as previously described¹⁵. Briefly 100 μ l of whole blood was stained with a biotinylated pig-specific antibody which does not cross react with baboon, followed by detection with Phycoerythrin Streptavidin and an APC-conjugated mouse anti-human CD45 clone D058-1283 (BD Biosciences). 1030H-1-10

positive cells not stained by anti-human CD45 mAb were considered to be of porcine origin. Acquisition was performed on a FACSCalibur and propidium iodide excluding cells within the lymphocyte gate analyzed using FloJo software. Standard deviation of the mean was calculated using Prism software.

Skin graft transplantation

Donor swine were premedicated with atropine 0.04 mg/kg and xylazine 1 mg/kg, and sedated with telazol 2 mg/kg IM, prior to maintenance anesthesia with inhaled isoflurane 1–5% in oxygen. Following antiseptic skin preparation with 2% (w/v) chlorhexidine acetate (Nolvasan Surgical Scrub, Fort Dodge Animal Health, Fort Dodge, IA), 70% isopropyl alcohol (Owens & Minor, Mechanicsville, VA) and 10% povidone-iodine (Betadine Solution, Purdue Products, Stamford, CT) split-thickness skin grafts were harvested from the flank using an air-driven dermatome (Zimmer U.K. Ltd, Wiltshire, U.K.). Grafts were immediately placed in 0.9% normal saline, and transferred directly to the recipient table for transplantation. Skin graft recipient baboons were premedicated with atropine 0.04mg/kg IM and sedated with ketamine 20mg/kg IM. Dorsal hair was shaved, and following endotracheal intubation, animals were placed in the prone position. Skin preparation was as above, and full-thickness recipient wound beds were prepared by excision of dorsal skin and subcutaneous tissue down to dorsal muscle, inferior to the level of the scapulae. Following meticulous hemostasis with electrocautery, and anchoring of the wound margins to underlying muscle with absorbable suture, skin xenografts and/or allograft controls were then transplanted and secured with simple interrupted nylon sutures, and absorbable quilting sutures. Grafts were dressed with bacitracin, nonadherent telfa gauze and adherent tegaderm to achieve compression and protection from shear forces. The animals were then placed in jackets to protect the grafts. Dressings were changed on posttransplant day 2 and alternate days thereafter, and sutures were removed on posttransplant day 14.

Skin graft assessment

Skin graft appearance was documented by serial photography and grafts determined to be rejected when less than 10% of the original graft remained viable. Periodic graft biopsies were performed under sedation and local anesthesia using a 3mm diameter biopsy punch. Biopsies were taken down to underlying muscle to ensure inclusion of full-depth graft tissue in each sample. Hemostasis and wound closure was achieved with 3/0 nylon suture dressings applied as described above. Specimens were fixed in 10% formalin, sectioned and stained with hematoxylin and eosin for assessment and scoring, unblinded, by a board certified pathologist.

Results

Variable Expression of the hCD47 Transgene in Genetically Identical Cloned Pigs

We previously reported the generation of a cloned transgenic miniature swine (18286) on an α -1,3-galactosyltransferase null (GalT-KO) background by recloning from a transgenic fetus (846-2)¹⁸. Both the fetus and 18286 demonstrated uniform expression of hCD47 on HC and fibroblasts. Despite recloning from the same fetus, a second transgenic pig (18289) demonstrated very low hCD47 transgene expression on peripheral blood mononuclear cells

(Figure 1A). Marked differences in transgene expression following cloning and nuclear transfer have been reported previously ²¹, although the basis for this phenomenon remains unclear. Nevertheless, the availability of these 2 otherwise identical animals offered us the unique opportunity to determine whether the level of expression of hCD47 on porcine HC would affect their clearance from the primate circulation.

hCD47 expression greatly increases the persistence of infused pig HC in primate peripheral blood

To test the hypothesis that expression of hCD47 would inhibit primate clearance of porcine HC, we transplanted cells from transgenic pigs 18286 and 18289 into baboons. Mobilized peripheral blood stem cells (PBSC) from these transgenic swine were utilized as the source of PBSC and cells from each animal were transplanted into 2 baboons conditioned with a preparative regimen that included low-dose whole body irradiation and lymphocyte depletion ²². Recipient baboons B337 and B342 received PBSC from the hCD47 high-expressing pig 18286, which demonstrated uniform expression of hCD47 in the mobilized PBSC population ¹⁸. Baboons B370 and B371 received cells from low-expressing pig 18289. Transplantation of PBSC was performed immediately following conditioning (Day 0) and additional challenge infusions from the same swine were administered 7 and 9 weeks later. Immunosuppression consisted of calcineurin inhibition and T cell costimulatory blockade with an anti-CD154 mAb for the first 4 weeks, anti-CD154 mAb only for the next 3 weeks and no immunosuppression thereafter. Both baboons receiving PBSC from donor 18286 demonstrated readily detectable macrochimerism in peripheral blood for several days following the initial cell transplant and following both subsequent cell infusions (Figure 1B,C). In contrast, chimerism was rapidly lost in the baboons receiving PBSC from donor 18289, and was undetectable by 1 day following infusion, consistent with previously reports of porcine hematopoietic cell clearance following infusion into primates ^{13–15}.

Prolongation of hematopoietic chimerism is associated with markedly prolonged survival of donor swine skin xenografts in the absence of immunosuppression

We next asked whether the HSC transplants might have affected recipient responses to tissue transplants from the donor swine. Split-thickness skin grafts (STSG) from the transgenic PBSC donors were placed onto full-thickness graft beds at 14 weeks after the initial cell transplants on the 4 baboons, by which time all exogenous immunosuppression had been discontinued for over 4 weeks. A control baboon (B360) received identical conditioning and immunosuppression regimens, but no cell transplants. Grafts were observed every 2–3 days and considered rejected when apparently viable tissue comprised less than 10% of the entire graft area.

The gross appearance of the skin grafts on baboons B337, B370 and B360 is shown in Figure 2. Grafts on all recipients were initially adherent and showed signs of vascularization and bruising over the first 4 days. The graft on B337, which received HSC transplants from high hCD47 expressing swine donor 18286, showed small islands of healthy tissue, which subsequently expanded and coalesced, to cover the entire area of the full-thickness graft bed with healthy porcine skin by day 42. We observed the continuing appearance of healthy porcine tissue covering the entire full-thickness graft bed, without contraction of the graft

bed, for over 53 days following STSG placement, at which point the entire graft had the appearance of normal, healthy porcine skin. The appearance of the graft deteriorated slowly over the following 2 weeks, reaching less than 10% viable epidermis by visual inspection on day 70. An allogeneic skin graft on B337 also showed prolonged survival but with progressive, marked contraction of the graft and graft bed and with histologic evidence of severe cellular rejection. The second recipient of cells and skin from the hCD47 uniform expressing donor (B342) followed a similar time course for the first 23 days post grafting, but then developed signs of local wound infection, leading to graft loss, with histologic evidence of bacterial colonization, by day 33. In marked contrast, grafts on both recipients of HSC transplants from low hCD47 expressing swine donor 18289 (B370 and B371) and the control recipient (B360) were judged clinically rejected by day 14.

Histological examination of the porcine graft on B337 at 53 days revealed completely normal dermal and epidermal structure, with only a mild, eosinophil-rich, cellular infiltrate in the dermis (Figure 3A). Biopsies on day 67 showed extensive dermal infiltration characteristic of grade 4 rejection and epidermal loss.

Consistent with their early rejection, biopsies from the low hCD47 recipients and the control recipient all showed complete loss of epidermis and extensive dermal necrosis accompanied by polymorphonuclear infiltration consistent with a necrotic process by day 10 (Figure 3B). In stark contrast, biopsies of high hCD47 recipients B337 and B342 at days 14 and 19 respectively revealed a well-maintained dermis with areas of reepithelialization and no cellular infiltrate. Capillaries with reactive endothelium were also observed in these recipients, but absent from the early rejecting recipients. We have no evidence to suggest that preexisting antibodies are involved in early rejection, as all recipients had at most barely detectable cytotoxic IgM levels, and no cytotoxic IgG, through the first 10–14 days following skin grafting (Supplementary Figures 1 and 2).

Discussion

These results indicate a strong correlation between hCD47 expression, prolonged peripheral porcine chimerism, and marked prolongation of porcine skin grafts in the absence of concurrent immunosuppression. Previous *in vitro* studies^{23;24} and *in vivo* murine studies^{18;25;26} with hCD47 expressing cells, as well as macrophage depletion studies in primates^{13–15}, strongly suggest that inhibition of reticuloendothelial engulfment is responsible for the prolonged chimerism, but direct demonstration of this in a primate model is difficult to obtain and awaits further studies. Since the control animal and all 4 recipients of PBSC had received the same immunosuppressive regimen and since skin grafts were placed more than 4 weeks after all immunosuppression had been discontinued, the most likely explanation for prolongation of skin graft survivals in B337 and B342 is a systemic immune modulating effect of the transient mixed chimerism in these recipients.

Dissection of the immune mechanisms involved in prolonged xenogeneic skin survival will require additional investigation. A detailed serial examination of histology in future recipients at the level of the graft bed over the first week may provide insight into the mechanism by which hCD47 high recipients B337 and B342 were able to avoid complete

epidermal loss and dermal necrosis. Understanding the mechanisms involved in late graft loss will also benefit from serial examination during the rejection period in future recipients. With the exception of an eosinophilic infiltrate, no suggestion of a rejection process was evident in biopsies of B337 at day 53 following grafting. The following biopsy, taken at the first overt clinical signs of graft degradation on day 67, revealed grade 4 rejection with extensive acute and chronic infiltration and as such was uninformative with respect to potential mechanisms of graft loss. Whether, in addition to prolonging peripheral chimerism, hCD47 has a role within the graft itself also needs to be examined. This can be best addressed in recipients conditioned with hCD47 high cells followed by simultaneous skin grafting from the hCD47 cell donor and a congenic, nontransgenic control. The inbred, MHC-defined miniature swine used in this study will therefore provide a unique opportunity to examine the role of graft expression of hCD47 in this manner.

Prolongation of skin graft survival is generally considered the most stringent test for immunosuppressive or tolerance-inducing regimens in the field of transplantation^{19;27;28}. While full durable tolerance was not achieved in this study, it nonetheless presents the first demonstration of the efficacy of hematopoietic cell transplantation in providing an immune modulating effect for porcine grafts transplanted to primate recipients. Previous primate studies have demonstrated good preservation of graft architecture and function for over 100 days following life-sustaining transplantation of porcine kidneys²⁹ and for over 1 year following heterotopic transplantation of porcine hearts³⁰. Graft survival in these cases required continuous administration of multiple immunosuppressive agents, including frequent infusion of T cell costimulation-blocking antibodies. Long-term graft maintenance under such conditions would likely be associated with high rates of serious or fatal infections and lymphoproliferative disease, limiting potential clinical applicability. In this study, recipients received prophylactic antibiotic and antiviral agents for the first month of conditioning, but required no therapeutic agents thereafter and in all cases maintained excellent health throughout the study. Thus, the 2-month survival of a porcine skin graft following the withdrawal of all immunosuppression reported here strongly suggests that hematopoietic cell conditioning may allow for long-term xenograft function in conjunction with a clinically acceptable maintenance regimen. If this result can be extended successfully to vascularized organ xenotransplants, it could greatly expand the number of life-sustaining organs available for transplantation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

GalT-KO	α -1,3-galactosyltransferase knock-out
HC	hematopoietic cells
hCD47	human CD47
IL-3	Interleukin-3
SCF	Stem Cell Factor
SIRP	Signal Regulatory Protein
STSG	split thickness skin graft
TBI	total body irradiation

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FIGURE 1.

hCD47 transgene expression in porcine cells correlates with prolonged peripheral chimerism in baboon recipients. A, hCD47 expression differs dramatically between genetically identical transgenic cloned swine. hCD47 expression on peripheral blood mononuclear cells from cloned transgenic swine 18286, 18289 and a WT control. Uniform expression of the transgene was observed in 18286, whereas expression was limited to a small fraction of PBMC in 18289. B, Peripheral blood levels of porcine leukocytes following transplantation into baboon recipients immediately following conditioning (1st Cell Infusion, Day 0), 7 weeks later (2nd Cell Infusion) and 9 weeks later (3rd Cell Infusion). B337 and B342 received PBSC from hCD47 transgenic donor 18286; B370 and B371 received PBSC from transgenic donor 18289. Day 0 samples were obtained 5 hours following the 1st cell infusion and 1 hour following the 2nd and 3rd cell infusions. C, Representative FACS analyses of Day 1 samples following the first cell infusion in B342 and in B371. Porcine cells were detected in whole blood staining as those staining positively with anti-pan swine monoclonal antibody 1030H-1-19 and negatively with primate anti-CD45 antibody. The absolute number of porcine leukocytes per ml was derived by multiplying the percent chimerism by the white blood cell count.

FIGURE 2.

Transient porcine chimerism leads to prolonged survival of porcine skin grafts on baboon recipients in the absence of immunosuppression. Top: Timeline of cell infusions, immunosuppressive therapy and skin grafting for these experiments. Bottom: Photographs of split thickness porcine skin grafts on the indicated day following graft placement. B337 and B342 received cell and skin transplants from hCD47 transgenic swine 18286 (high hCD47 expression), while B370 received cells and skin from swine 18289 (low hCD47 expression). B360 received the same conditioning regimen and a skin graft from 18289, but no cell transplants. All grafts initially showed signs of vascularization (marked by bruising). In recipient B337, healthy appearing islands of porcine skin gradually expanded to fill the entire graft bed. B342 showed a graft course similar to that observed in B337, but the graft was lost to infection prior to substantial epidermal expansion. In contrast, B370 and B360 completely rejected their grafts within 10–14 days.

Figure 3.

A, Histological examination confirms the reestablishment of normal dermal and epidermal structure at 53 days after porcine skin transplantation. H&E staining of the porcine graft on B337 53 days following transplantation revealed some eosinophilic infiltration, but no classical signs of rejection. Biopsy on day 67 revealed extensive dermal acute and chronic inflammation with epidermal loss. B, Reepithelialization and maintenance of dermal viability in recipients B337 and B342. H&E staining of biopsies from hCD47 high recipients B337 and B342 at days 14 and 19 respectively revealed areas of re-epithelialization (arrows), including migration along a hair follicle in B342 (inset). No cellular infiltration was observed in the graft for either recipient. In contrast, complete loss of epidermis, with dermal necrosis and cellular infiltration was seen in B370 and B360 at days 10 and 7 respectively.