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RECIPIENT TISSUE FACTOR EXPRESSION IS ASSOCIATED WITH CONSUMPTIVE COAGULOPATHY IN PIG-TO-PRIMATE KIDNEY XENOTRANSPLANTATION

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Abstract

Consumptive coagulopathy (CC) remains a challenge in pig-to-primate organ xenotransplantation (Tx). The present study investigated the role of tissue factor (TF) expression on circulating platelets and peripheral blood mononuclear cells (PBMCs). Baboons (n=9) received a kidney graft from pigs that were either wild-type (n=2), α 1,3-galactosyltransferase gene-knockout (GT-KO; n=1), or GT-KO and transgenic for the complement-regulatory protein, CD46 (GT-KO/CD46, n=6). In the baboon where the graft developed hyperacute rejection (n=1), the platelets and PBMCs expressed TF within 4h of Tx. In the remaining baboons, TF was detected on platelets on post-Tx day 1. Subsequently, platelet-leukocyte aggregation developed with formation of thrombin. In the 6 baboons with CC, TF was not detected on baboon PBMCs until CC was beginning to develop. Graft histopathology showed fibrin deposition and platelet aggregation (n=6), but with only minor or no features indicating a humoral immune response (n=3), and no macrophage, B or T cell infiltration (n=6). Activation of platelets to express TF was associated with the initiation of CC, whereas TF expression on PBMCs was concomitant with the onset of CC, often in the relative absence of features of acute humoral xenograft rejection. Prevention of recipient platelet activation may be crucial for successful pig-to-primate kidney Tx.

Keywords

Baboon; Coagulation; PBMC; Pig; Platelets; Tissue factor; Xenotransplantation

INTRODUCTION

Intravascular thrombosis and systemic coagulation disorders (consumptive coagulopathy [CC]) during acute humoral xenograft rejection (AHXR) have been major hurdles to successful xenotransplantation (Tx). Following heterotopic heart Tx from GT-KO pigs to

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baboons, all grafts failed from thrombotic microangiopathy with platelet-rich fibrin thrombi in the microvasculature, myocardial ischemia and necrosis, and focal interstitial hemorrhage (1). The expression of tissue factor (TF) on the graft endothelial cells (EC) occurred in parallel with EC activation. These and other data suggested that CC is initiated by the expression of TF in the graft(2-3), and correlates with previous studies indicating that TF expression resulted from activation of the ECs in response to deposition of xenoreactive antibodies (Abs) and complement activation (4-6).

TF binds factor VII/factor VIIa (FVII/VIIa), and the complex TF-FVIIa activates FX and FIX to initiate coagulation (7-8). In sepsis models, ECs and monocytes constitute the main origins of TF(9-11). TF is also expressed on granulocytes in different disease states (12-13). Microparticles shed from ECs or monocytes are the main source of circulating TF, and transfer TF to platelets (14-15). Recently, platelets have been shown to be capable of synthesizing and expressing functional TF by pre-mRNA splicing (16-17).

However, coagulation dysregulation can be seen not only in the graft; several studies reported that AHXR is associated with systemic coagulation cascade activation, consistent with non-physiologic disseminated intravascular thrombosis. This systemic CC is compatible with that seen in various conditions, e.g., endotoxemia, following cardiac surgery, and in cancer (18-19). Its pathophysiology may be associated with liberation of TF into the blood and inducible TF expression on leukocytes and other cells. A similar situation occurs in islet allo- and xeno-transplantation following their infusion into the portal vein. Many islets are destroyed immediately from the 'instant blood-mediated inflammatory reaction', which is associated with TF expression on endocrine cells (20-21). Berman et al showed that interference with TF activity by using a humanized anti-TF Ab leads to prolonged and/or enhanced islet allograft function in non-human primates (22).

Our previous *in vitro* study showed that, after contact with porcine aortic endothelial cells (PAECs), human platelets and monocytes are activated to express TF, and this is associated with increased TF activity (23). We hypothesize that, following pig organ Tx into primates, recipient platelets and leukocytes are activated to express functional TF and may be a causative factor in the development of CC. We have explored this hypothesis in the present study.

MATERIALS AND METHODS

Animals

Baboons (*Papio* species, 6–20 kg, of known ABO blood type [n=9], Division of Animal Resources, Oklahoma University Health Sciences Center, Oklahoma City, OK) were recipients of pig kidney grafts.

Wild-type (WT; n=2), α 1,3-galactosyltransferase gene-knockout (GT-KO, n=1)(24), or GT-KO pigs transgenic for the complement-regulatory protein, CD46 (GT-KO/CD46, n=6) (all from Revivicor, Inc., Blacksburg, VA) were sources of kidneys; all were of blood group O (non-A). GT-KO was confirmed by negative expression of biotinylated GSIB4 lectin in major organs (24). High CD46 expression was confirmed on PAECs and peripheral blood mononuclear cells (PBMCs) by flow cytometry(25).

All animal care was in accordance with the Principles of Laboratory Animal Care (NIH publication No. 86-23, revised 1985). Protocols were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Surgical procedures, supportive therapy, and monitoring

Anesthesia, kidney harvesting in pigs, and renal Tx in baboons have been described previously (26-27). Both native baboon kidneys were excised. All recipient baboons received prophylactic intravenous (i.v.) antibiotics for the first 3 post-Tx days, and famotidine and ganciclovir throughout the post-operative period. Conventional heparin was begun on day 0 (day of Tx) and gradually increased in an effort to maintain the activated partial thromboplastin time (APTT) at approximately 150sec from day 3, as described previously (26), but was discontinued when the recipient developed features of CC, particularly if spontaneous bleeding occurred. Human albumin was administered i.v. from day 0 to prevent hypoalbuminemia that resulted from persistent proteinuria. Washed baboon red blood cells were administered when necessary to maintain the hematocrit >20%.

Blood cell counts, chemistry, and coagulation parameters (prothrombin time, APTT, fibrinogen, D-dimer) were measured in the Central Laboratory of the University of Pittsburgh Medical Center. Kidney graft status was monitored by serum creatinine. CC was defined by the development of the following features: (i) unexplained and persistent thrombocytopenia (>30% fall in platelet count) and (ii) significant and persistent decrease in serum fibrinogen (and increase in serum D-dimer), followed by (iii) clinical lethargy, requiring euthanasia, and/or (iv) overt bleeding, requiring euthanasia.

Immunosuppressive therapy

Two baboons received grafts from WT (n=1) or GT-KO/CD46 (n=1) pigs without immunosuppressive therapy (Table 1). Immunosuppressive therapy was administered to 7 baboons that received grafts from WT (n=1), GT-KO (n=1), or GT-KO/CD46 (n=5) pigs with immunosuppressive therapy (Table 1). Immunosuppressive therapy varied, but was based on induction with anti-thymocyte globulin and maintenance with a human anti-CD154 monoclonal Ab (mAb; ABI193, a generous gift from Novartis Pharma, Basel, Switzerland), and mycophenolate mofetil (MMF, Roche, Nutley, NJ), with or without methylprednisolone. Cobra venom factor (CVF) was administered to two baboons (Table 1).

Preparation of platelets, PBMCs, and leukocytes

Baboon blood was collected into tubes containing ethylenediaminetetraacetic acid (EDTA, 5 mM) or acid-citrate-dextrose (ACD). After centrifugation (10min at 150g), the top two-thirds of platelet-rich plasma was removed and centrifuged (8min at 1400g). The pellet was washed with buffer (137mM NaCl, 5.3mM KCl, 1mM MgCl₂, 2mM CaCl₂, 4.1mM NaHCO₃, and 5.5mM glucose [pH 6.5]) containing 120nM prostaglandin E₁ (Sigma, St Louis, MO). Platelets were centrifuged (5min at 150g) to remove residual leukocytes. Platelets were kept at 4°C during the isolation steps to avoid activation. Baboon PBMCs and leukocytes were isolated as previously described (28).

Recalcified clotting assay

Functional TF activity was determined by a recalcified clotting assay. Baboon platelets (5×10^6) or PBMCs (1×10^6) were suspended in 50μl Tris-buffered saline and mixed with 100μl of Factor VII (FVII)-deficient human plasma (Haematologic Technologies, Essen Junction, VT) in glass tubes (Corning, Corning, NY). One hundred microliters (100μl) of 25mM CaCl₂ in Tris-buffered saline were added and the tube incubated at 37°C in a water bath; the time for a fibrin clot to form was measured, during which time the tubes were continuously agitated by tilting. In these assays, TF-dependent thrombin generation requires the participation of FVII; the clotting time is shortened when FVII is added. Therefore, the procedure was repeated with the addition of FVII (Haematologic Technologies). The

activity of TF was determined by a comparison (ratio) of the clotting times measured with/without FVII. In each assay, the clotting time was determined in triplicate, and the results were quantified from a standard curve prepared by a series of dilutions of soluble recombinant human TF (R&D, Minneapolis, MN) and expressed as a procoagulant activity equivalent to nanograms (ngs) of human TF.

Platelet-leukocyte aggregation assay

Baboon leukocytes were stained by anti-CD42a- FITC (BD Pharmingen, San Jose, CA) and anti-CD45-phycoerythrin (PE)(BD Pharmingen) for analysis by flow cytometry. Anti-mouse IgG1-FITC (Serotec, Raleigh, NC) and anti-mouse IgG1-PE (Serotec) were used as isotype controls. Lymphocytes, monocytes, and polymorphonuclear populations were differentiated by forward and side scatter. The percentages of the leukocyte subgroups co-expressing CD42a (platelets) and CD45 (leukocytes) were determined and used as an index of leukocyte-platelet aggregation.

Baboon or human IgM and IgG antibody binding to PAECs

Cells (10^5) were incubated with 20% heat-inactivated serum or FACS buffer (control) for 30min at 4°C. Detection of IgM or IgG binding was performed by further incubating the serum with FITC-conjugated goat anti-human IgM (μ chain-specific) and IgG (γ chain-specific) Abs (Invitrogen, Camarillo, CA) for 30min in the dark at 4°C. Binding of xenoreactive Abs to PAECs was determined by flow cytometry (28).

Immunohistology of pig kidney grafts

Biopsies of the pig kidney grafts were obtained (i) before graft implantation and (ii) at the time of euthanasia (Table 1). Tissues were fixed in 10% formalin and embedded in paraffin. Sections (4mm) were stained with hematoxylin-eosin and with martius-scarlet-blue for light microscopy. Immunohistochemical staining for IgM, IgG, C3, C4d, fibrin and platelets, neutrophils (myeloperoxidase), macrophages (CD68), T lymphocytes (CD3), and B lymphocytes (CD20) was performed.

Cryostat sections of xenograft kidneys were fixed and incubated with sheep anti-human TF Ab, mouse anti-porcine E selectin (clone 1.2B6, Sigma) and mouse anti-human CD42 or CD45 mAbs (Serotec), followed by CyChrome 2 anti-sheep IgG and CyChrome 3 anti-mouse IgG secondary Abs (Jackson ImmunoResearch, West Grove, PA). Nuclei were stained with DAPI (4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Images were analyzed with a Leica Microsystems TCS-NT microscope (Melville, NY).

Statistical analysis

Data are presented as mean $\pm$ SEM. Significance of the difference between two groups was determined by paired Student's t-test. Values of $P < 0.05$ were considered significant.

RESULTS

Outcome of pig kidney transplantation in baboons

The baboon that received a WT kidney graft without immunosuppression developed acute renal failure (rapidly increasing serum creatinine) within 24h, resulting in a need for euthanasia (Table 1); the graft revealed gross thrombotic changes compatible with the typical features of HAR (not shown).

In all other experiments, HAR was prevented either by using a kidney from a genetically-modified pig or by the administration of agents to inhibit the humoral response. Two

baboons died of transplant-unrelated causes 2 and 4 days after Tx; iatrogenic fluid and albumin overload was a likely causative factor (Table 1). Hematological and biochemical profiles remained within normal limits, and there were no significant abnormal findings at necropsy.

The remaining 6 recipients survived for 6-16 days (median 9.5 days) and either died or were euthanized for features of CC (Table 1). Features of CC initially developed between days 3 and 8 (median 6) at which time there was a fall in platelet count which was progressive (Figure 1). Subsequently, CC progressed with bleeding at the site of the surgical wound or melena stools being common (n=4), requiring blood transfusion.

TF expression and activity on recipient platelets and PBMCs

TF expression on baboon platelets and PBMCs was confirmed by flow cytometry and quantitative RT-PCR (supporting methods) with a baboon, but not a porcine, TF primer (Figures 2A,B). In the baboon that hyperacutely rejected the pig kidney, platelets and PBMCs expressed TF 4h after Tx (Figure 2C). In the grafts in the 2 baboons that died from fluid overload in the absence of features of rejection or CC, TF expression on platelets was demonstrated on day 1. In the remaining 6 baboons that developed CC, TF expression was demonstrated on platelets at 1-2 days and on PBMCs on days 3-6, which just preceded the development of features of CC (Figure 2D).

TF activity on platelets and PBMCs was determined by the recalcified clotting assay. The increased TF activity on platelets and PBMCs was associated with baboon TF, but *not* porcine TF, since it was inhibited by an anti-human TF Ab. Because of a different avidity between the species, this Ab inhibits only human, but not pig, TF activity in the clotting assay (23) (Figure 2E).

TF expression and activity on pig kidney grafts and native baboon organs

At death or euthanasia of those baboons with CC (n=6), immunofluorescent staining of the pig kidney graft showed that TF was expressed on the platelet aggregates in the clot and on PBMCs (Figure 3A). Consistently, baboon TF activity was demonstrated by quantitative RT-PCR in the porcine grafts (Figure 3B).

Since TF activity on platelets and PBMCs may cause systemic effects, TF activity in the major native organs of the recipients was investigated. Using heart and liver tissues from other (naïve) baboons as controls, the native hearts and livers of the kidney xenograft recipients were demonstrated to express significantly higher levels of TF mRNA with baboon TF primer, but not the porcine TF primer (Figure 3C). In contrast, porcine TF activity in the grafts was not significantly different (by quantitative RT-PCR) from that seen in the donor pig before kidney Tx (Figure 3B). At euthanasia, the endothelium of 3 of 6 porcine kidney grafts revealed minimal activation, evidenced by the expression of E selectin (Figure 3D).

TF expression on platelets is associated with platelet-leukocyte aggregation

Since TF expression on PBMCs developed later than on platelets, we investigated the interaction between platelets and PBMCs. Leukocytes were identified as CD45-positive; granulocytes, monocytes, and lymphocytes were differentiated on flow cytometry by forward and side scatter (Figure 4A). The respective platelet-granulocyte, -monocyte, or -lymphocyte aggregates were significantly greater on day 2 (when TF was first expressed on platelets) than before Tx (Figure 4B), and increased gradually with time (Figure 4C). Platelet-leukocyte aggregation dramatically increased when PBMCs demonstrated increased TF activity and CC developed.

Serum thrombin formation and kidney function after expression of TF on platelets

To examine the effect of TF expression on the development of thrombosis, sequential serum fibrinogen levels and D-dimer were measured. D-dimer levels were slightly increased at the time of initial TF expression on platelets; thereafter, the levels rose significantly to a plateau by the time of TF expression on PBMCs (Figure 5A). Similarly, serum fibrinogen levels began to fall when TF became expressed on platelets; the lowest levels were reached when TF became expressed on PBMCs and CC developed (Figures 5B,C). Importantly, the graft remained functioning well throughout this process (evidenced by adequate urine output and normal serum creatinine levels) (Figures 5B,C). In B16007, the serum creatinine remained in the normal range for another 7 days after the development of profound thrombocytopenia (Figure 5C). All of the recipients (n=6) succumbed to, or were euthanized for, the systemic complications of CC, and not through renal dysfunction.

Immune responses

Serum anti-porcine (Gal and nonGal) and anti-nonGal IgG and IgM Abs were measured to determine the humoral immune response. Compared to pre-Tx sera, there was no change in the levels of serum anti-porcine and anti-nonGal IgG and IgM Abs throughout the post-Tx course (Figure 6A), indicating a lack of T cell-dependent sensitization to pig antigens.

Histology of pig kidney grafts

The histopathologic findings of the excised pig kidney grafts at euthanasia of the recipients with CC (n=6) are summarized Table 2. Of importance, in contrast to the kidney that underwent HAR (Figure 6B), 3 of 6 kidneys showed features of only a minimal humoral response (Figure 6B). In contrast to the typical features when a pig kidney has undergone AHXR, there was only minimal IgM deposition with no detectable deposition of IgG or C3, although there was deposition of fibrinogen. Infiltration with macrophages, B and T (both CD4 and CD8) cells was not seen in any kidney graft (Figure 6B).

DISCUSSION

Previous reports have suggested that CC is associated with the development of AHXR and is initiated by the expression of TF in the porcine graft (2-3,29). It was believed that donor ECs of the graft, activated by the binding of xenoreactive Abs and/or complement, initiated the development of AHXR and, consistently, the expression of TF. The increased TF activity initiated intragraft thrombosis and CC(29). The activated ECs and the generated thrombin subsequently activated platelets, leukocytes, and other inflammatory cells in the recipient, initiating a vicious cycle. However, the source of the TF, although presumed to be porcine in origin, remained uncertain. Determining the origin of the TF that initiates the development of CC is essential if steps are to be taken to prevent this complication.

In the present study, we demonstrated that, following pig-to-baboon kidney Tx, it was baboon platelets and PBMCs, rather than porcine EC, that were activated to express functional TF. This was associated with the development of CC. These findings are compatible with those from a rodent study in which inflammatory cells, especially platelets and mononuclear cells, activated by soluble inflammatory mediators or other immune response-independent pathways, stimulated coagulation through expression of TF (4).

TF activity on platelets was expressed early after kidney Tx, and, in the absence of HAR, the timing of expression was unaffected by the graft type (e.g., the extent of genetic modification of the pig) or by the intensity of the immunosuppressive regimen. However, this regimen was not as intense as others in which CC has not been reported (27), and therefore the intensity of the regimen may influence the outcome. TF expression on PBMCs

occurred later, and was closely associated with the onset of CC (e.g., the development of thrombocytopenia). This is similar to the development of disseminated intravascular coagulation in sepsis, in which inducible TF is predominantly expressed by monocytes and macrophages, as demonstrated in patients with severe bacterial infection (30). Recent studies have consistently indicated that leukocytes (e.g., monocytes, dendritic cells), activated by thrombin, play a central role in the inflammatory response and determine the severity of the disseminated intravascular coagulation that results (31).

The critical question is what factors activate PBMCs to express functional TF and induce CC. There are some possible explanations. First, it was previously thought that activated graft PAECs led to activation of primate PBMCs to express TF via upregulation of adhesion molecules such as P- and E-selectin or the effect of thrombin, which is formed during PAEC activation and is a potent stimulator of PBMCs. This process is thought to be associated with the immune response to the PAECs (5,29). Second, as our previous *in vitro* studies showed, after contact with porcine ECs, PBMCs are activated to express TF through a humoral response-independent pathway after contact with ECs (23,32). Third, TF expression on PBMCs is induced by activated platelets, which are activated at an earlier time-point. Activated platelets stimulate leukocytes to TF activity through interaction between P-selectin and PSGL-1 (33-34).

In the present study, we found that the serum level of fibrinogen began to fall, with a concomitant increase in the level of D-dimer, after TF became expressed on recipient platelets. Moreover, platelets and PBMCs progressively aggregated until CC developed. A recent study demonstrated that blood-borne TF contributes to thrombus propagation (35). Activated platelets can serve as inflammatory cells that activate leukocytes, and play vital roles in thrombosis and inflammation by 'cross-talk' between platelets and leukocytes (23,33). Our findings indicated that recipient platelets became activated to express TF early after Tx which, in turn, affected the coagulation cascade and was involved in activating leukocytes to induce CC.

In contrast, ECs appeared to remain inactivated. Since genetically-modified kidneys were used and were combined with the administration of a co-stimulation blockade agent (anti-CD154 Ab), the recipients did not become sensitized after Tx (evidenced by no increase in anti-porcine or anti-nonGal Abs). This suggests that ECs can be activated *without* sensitization, and this correlates with our previous *in vitro* studies(23). Furthermore, in 2 baboons (B3907, B18308) complement activity was eliminated by the administration of CVF (evidenced by the CH50 test, data not shown), and in 4 baboons the grafts were protected from complement activity by the over-expression of a human complement-regulatory protein (CD46), yet CC still developed. Importantly, the histology of 3 of the grafts showed minimal evidence of an immune response (with minimal deposition of IgM, without deposition of IgG or complement, and with no infiltration of macrophages, B, or T cells). In addition, the kidney xenografts still functioned satisfactorily (as indicated by serum creatinine levels) even after the development of CC. These observations suggest that the development of CC was not directly associated with the presence of an immune response.

Anti-CD154 Ab was suspected to induce thromboembolism in kidney transplantation(36). However, the complication could be reduced by administration of heparin. Moreover, in our liver xenoTx study (manuscript preparation), all recipients developed CC even without the use of anti-CD154 Ab. We believed that CC in the present study was not attributed to the complication of anti-CD154 Ab.

Taken together, although we cannot exclude that graft ECs became minimally activated, our results suggest that recipient activated platelets, *not* donor ECs, were associated with the

formation of thrombin, stimulating the activation of leukocytes and the initiation of CC. In support of this conclusion, porcine von Willibrand factor on ECs has been shown to activate primate platelets without the requirement of shearing force(37-38). Furthermore, pigs deficient in von Willibrand factor provided a survival benefit in a lung xenoTx model(39).

There are several questions that require further investigation. First, what exactly constitute the platelet and PBMC aggregates? Evidenced by CD42a expression, these aggregates may consist of cells or microparticles associated with PBMCs. Second, how do activated leukocytes induce CC? A recent study indicated that cell-derived microparticles (not parent cells) aggregate platelets through a TF-dependent pathway(40). Third, why was CC *not* reported in a pig-to-baboon kidney Tx model using an immunosuppressive regimen aimed towards tolerance induction (that included thymectomy, splenectomy, and T cell depletion) (27,41)? It may be that this intensive regimen not only induced a degree of lymphocyte unresponsiveness but, although graft survival was prolonged, interfered with the normal functions of leukocytes, as suggested by the high incidence of infection seen in the study. The relationship between immunosuppressive therapy, aimed largely at suppressing the adaptive immune response, and coagulation dysregulation is complex.

One other observation made in this study is worthy of brief comment, and that is that severe proteinuria (4+) developed in all of the baboons within hours of pig kidney Tx (and bilateral native nephrectomy). In the absence of clear evidence for an immune response (as indicated in the early post-Tx renal biopsies), this would suggest that the proteinuria was a result of a 'physiologic' rather than an immunologic problem. Massive proteinuria in baboons with pig kidney transplants has been reported previously by other groups (27,42-43). If it results from an intrinsic anatomic/physiologic difference between pig and primate kidneys (or possibly from an early effect of the innate - rather than the adaptive - immune response), proteinuria may be difficult to prevent.

In summary, we provide evidence that it is TF expression on recipient platelets and PBMCs that initiates CC after pig-to-baboon kidney Tx. We believe that TF expression on platelets plays a pivotal role in the induction of CC. This TF expression and activity on platelets would appear to be unrelated to the recipient's immune response since they occur when evidence for the response is minimal or absent. Further understanding of the interaction between porcine ECs and primate platelets by non-immunologic processes should be sought, perhaps by the use of organs from pigs that have undergone other genetic modifications or by systemic medication. Further manipulation of the immune response alone, with the risk of inducing infection and other complications, does not appear likely to resolve the challenge of xenograft coagulopathy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Ab(s)	antibody(ies)
AXHR	acute humoral xenograft rejection
CC	consumptive coagulopathy
ECs	endothelial cells
Gal	Gal α 1,3Gal
GT-KO	α 1,3-galactosyltransferase gene-knockout
HAR	hyperacute rejection
PBMCs	peripheral blood mononuclear cells
PCR	polymerase chain reaction
TF	tissue factor
Tx	xenotransplantation
WT	wild-type

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Figure 1. Platelet counts and serum creatinine levels in baboon recipients of pig kidney grafts B3807 developed HAR with a rapidly increasing creatinine level, but its platelet count remained unchanged. Two baboons (B4407, B4507) died of causes unrelated to AHXR or CC. The remaining 6 baboons developed features of CC (defined in the text). Arrows with numbers indicate the day on which CC was initially documented. (The day of kidney Tx was designated as day 0). HAR = hyperacute rejection; CC = consumptive coagulopathy, black line: platelet counts, gray line: serum creatinine level.

Figure 2. TF expression and activity on recipient platelets and PBMCs

TF expression and activity were measured on baboon platelets and PBMCs before and after pig kidney Tx. TF expression was confirmed by (A) flow cytometry (gray: isotype control) in all recipients, and (B) quantitative RT-PCR (B3807, in which HAR occurred, * $P < 0.05$). (C) The day when TF expression was detected on baboon platelets (closed circles, $n=9$) and PBMCs (open circles, $n=7$). (D) Relationship between the first day of TF expression on PBMCs (white circles, $n=1$) and the first day of features indicating the development of CC (black circles, $n=2$). (E) TF activity was determined by the recalcified clotting assay. Clotting assays were performed before (white bar) and after (black bar) incubation of platelets or PBMCs with anti-human TF Ab (* $P < 0.05$ compared to pre-Tx).

D

Figure 3. TF expression in the pig kidney grafts and native baboon organs

(A) TF expression on platelets and PBMCs in the graft was examined by immunofluorescent staining. Grafts were stained with TF (green) and either CD42a (platelets, red, 400X) or CD45 (PBMCs, red, 400X), respectively. TF expression on platelet aggregates (upper two panels) and PBMCs (lower two panels) within the graft is shown pre-Tx and at euthanasia (Euth). (Representative results from B18303).

(B) Baboon (white) and porcine (gray) TF mRNA expression in the pig kidney grafts (left to right – naïve pig kidney (n=6), naïve baboon kidney (n=8), the pig kidney that underwent HAR (n=1), and the kidneys in the baboons that developed CC (n=6). (* $p < 0.05$ baboon TF expression in the pig grafts versus TF expression on naïve baboon kidneys).

(C) Baboon TF expression at euthanasia in the native hearts (left panel) and livers (right panel) of the baboons that received pig kidney grafts, compared to TF expression in naïve baboon organs (* $p < 0.05$).

(D) E selectin expression on porcine kidney grafts by immunofluorescent staining. The expression of E selectin (red, 200X) was minimal in 3 baboons that developed CC (represented by B16007, right) compared with expression in the pig kidney graft that underwent HAR (left).

A **B**

Figure 4. TF expression on platelets is associated with platelet-leukocyte aggregation

(A) In dot plot, there were 3 distinctive groups of cells - lymphocytes (L), monocytes (M), and granulocytes (G). (B) The percentages of CD42a-positive cells (platelets) in the granulocyte (upper), monocyte (middle), and lymphocyte (lower) gates before Tx, on day 2 (first day of TF expression on platelets), and day 6 (day of onset of CC). The percentage of cells in each quadrant is indicated. (Representative data from B18308) (C) The time course of correlation between platelet count (indicating the development of CC), TF expression on platelets and PBMCs, and platelet-leukocyte aggregation in a representative baboon (B18308).

Figure 5. Correlation of thrombotic profile, serum creatinine, and TF expression after pig kidney Tx in baboons

(A) Serum D-dimer levels pre-Tx (open bar) and on the days of TF expression on baboon platelets (black bar) and PBMCs (gray bar). (* $P < 0.05$, compared to pre-TX)

(B) Mean platelet count, TF expression on platelets and PBMCs, serum fibrinogen, and serum creatinine in 4 baboons with pig kidney grafts (B18308, B4207, B4307, B16307).

(C) Platelet count, TF expression on platelets and PBMCs, serum fibrinogen, and serum creatinine in the baboon with the longest survival (16 days) (B16007).

Figure 6. Serum IgM and IgG levels and histopathologic features of kidney grafts

(A) Ratio of mean fluorescence intensity (MFI) of serum anti-porcine (left panel) and anti-nonGal (right panel) IgM (black bars) and IgG (blank bars) levels pre-Tx (day -1) and at intervals until euthanasia (measured by flow cytometry). Pre-Tx (day -1) was scored as 1. MFI ratio indicates the MFI at each time-point divided by the MFI of the pre-Tx sample in each baboon.

(B) Deposition of (a) IgM (brown, 200X), (b) IgG (brown, 200X), (c) C3 (brown, 200X), and (d) fibrin (red, 400X) in the pig kidney graft that underwent HAR (B3807). In contrast, the remaining pig kidney grafts showed minimal deposition of (e) IgM (400X), no deposition of (f) IgG (400X) or (g) C3 (400X), but some deposition of (h) fibrin (600X), with no infiltration of (i) macrophages (CD68, 100X), (j) B cells (CD20, 100X), (k) CD4 cells (100X), or (l) CD8 cells (100X). (Findings in a representative baboon, B4207)

Table 1

Graft type, immunosuppressive therapy, outcome, graft survival, and cause of death or euthanasia in pig-to-baboon kidney transplantation.

#	Type of pig	Immunosuppressive regimen	Survival (days)	Cause of death / euthanasia
B3807	WT	None	<1	HAR
B4407	GT-KO/CD46	None	2	Fluid overload
B4507	GT-KO/CD46	ATG, Anti-CD154 mAb, MMF, MP	4	Fluid overload
B3907	WT	ATG, Anti-CD154 mAb CVF, MMF, MP	6	CC
B18308	GT-KO	ATG, Anti-CD154 mAb CVF, MMF, MP	7	CC
B4207	GT-KO/CD46	ATG, Anti-CD154 mAb MMF	9	CC
B4307	GT-KO/CD46	ATG, Anti-CD154 mAb MMF	10	CC
B16307	GT-KO/CD46	ATG, Anti-CD154 mAb MMF	10	CC
B16007	GT-KO/CD46	ATG, Anti-CD154 mAb MMF	16	CC

ATG= Anti-thymoglobulin, CC = Consumptive coagulopathy; CVF = Cobra venom factor; HAR = hyperacute rejection; MMF = mycophenolate mofetil, mAb= monoclonal antibody, MP= methylprednisolone.

Table 2

Deposition of immunoglobulin, complement, and fibrinogen, and infiltration by inflammatory cells in pig kidney grafts in baboons that developed hyperacute rejection or consumptive coagulopathy.

	IgM	IgG	Fibrin	C3	MΦ	PMN	B cells	T cells
B3807	(++)	(++)	(+++)	(+++)	(++)	(++)	(+)	(+)
B3907	(+)	(+)	(+++)	(--)	(--)	(--)	(--)	(--)
B18308	(+)	(+)	(+++)	(--)	(--)	(--)	(--)	(--)
B4207	(+/-)	(--)	(++)	(--)	(--)	(--)	(--)	(--)
B4307	(+/-)	(--)	(++)	(--)	(--)	(--)	(--)	(--)
B16307	(+/-)	(--)	(+++)	(--)	(--)	(--)	(--)	(--)
B16007	(++)	(++)	(+++)	(++)	(--)	(--)	(--)	(--)

(--) none; (+/-): minimal; (+) mild; (++) moderate; (+++) severe.

MΦ = macrophages; PMN = polymorphonuclear neutrophils