ORIGINAL ARTICLE

Potential factors influencing the development of thrombocytopenia and consumptive coagulopathy after genetically modified pig liver xenotransplantation

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Summary

Upregulation of tissue factor (TF) expression on activated donor endothelial cells (ECs) triggered by the immune response (IR) has been considered the main initiator of consumptive coagulopathy (CC). In this study, we aimed to identify potential factors in the development of thrombocytopenia and CC after genetically engineered pig liver transplantation in baboons. Baboons received a liver from either an α1,3-galactosyltransferase gene-knockout (GTKO) pig ($n = 1$) or a GTKO pig transgenic for CD46 ($n = 5$) with immunosuppressive therapy. TF exposure on recipient platelets and peripheral blood mononuclear cell (PBMCs), activation of donor ECs, platelet and EC microparticles, and the IR were monitored. Profound thrombocytopenia and thrombin formation occurred within minutes of liver reperfusion. Within 2 h, circulating platelets and PBMCs expressed functional TF, with evidence of aggregation in the graft. Porcine ECs were negative for expression of P- and E-selectin, CD106, and TF. The measurable IR was minimal, and the severity and rapidity of thrombocytopenia were not alleviated by prior manipulation of the IR. We suggest that the development of thrombocytopenia/CC may be associated with TF exposure on recipient platelets and PBMCs (but possibly not with activation of donor ECs). Recipient TF appears to initiate thrombocytopenia/CC by a mechanism that may be independent of the IR.

Introduction

Intravascular thrombosis and systemic consumptive coagulopathy (CC) in the presence or absence of features of acute humoral xenograft rejection (AHXR) remain hurdles for successful pig-to-primate organ transplantation (TX). For example, in the model of heterotopic cardiac TX from α1,3-galactosyltransferase gene-knockout

(GTKO) pigs to baboons, while graft survival was prolonged when compared with wild-type (WT) pig xenografts, and no Gal-mediated hyperacute rejection (HAR) was observed, ultimately all grafts failed owing to the development of thrombotic microangiopathy with platelet-rich fibrin thrombi in the microvasculature, myocardial ischemia and necrosis, and focal interstitial hemorrhage [1].

However, the mechanism by which coagulation disorders develop after xenotransplantation remains elusive. Previous reports suggested that CC is initiated by the expression of tissue factor (TF) in the porcine graft [2,3]; in response to the binding of xenoreactive antibody and/ or activation of complement, endothelial cells (ECs) in the graft are activated to increase TF activity and to initiate intragraft thrombosis and CC [4,5].

During inflammation, type I activation of ECs is mediated by ligands binding to the extracellular domains of G protein-coupled receptors, inducing display of P-selectin and vascular leakiness of plasma proteins [6,7]; this process takes 10–20 min. Type II activation of ECs is triggered by stimulation of tumor necrosis factor-α and interleukin-1, induces more effective leukocyte recruitment by synthesis of adhesion proteins, such as E-selectin and CD106 [vascular cell adhesion molecule-1 (VCAM-1)], and is sustained for 6–24 h after cytokine-mediated activation [7,8]. Type I and type II activations are usually believed to be associated with HAR and AHXR, respectively [4]. The activated ECs and the generated thrombin subsequently activate platelets, leukocytes, and other inflammatory cells in the recipient, initiating a vicious cycle.

In contrast, our previous *in vitro* results indicated that porcine aortic endothelial cells (PAECs) are able to induce human TF on human platelets and monocytes through an immune response-independent pathway [9]. This observation suggested that additional manipulation of the immune response (with the increased risks of infection and other complications) will not completely overcome CC after xenotransplantation. Hence, it is important to determine the mechanism by which CC is initiated after xenotransplantation because it may enable additional genetic modification of the pig or suggest therapy that might prevent CC.

In our reported studies [10,11], hepatic function after genetically engineered pig liver xenoTX was in the near-normal range, except for some cholestasis, as demonstrated by measurements of liver enzymes, coagulation parameters, and factors using conventional methods, and porcine-specific proteins (albumin, fibrinogen, haptoglobin, and plasminogen) using Western blot [10,11]. However, thrombocytopenia developed within minutes after reperfusion of the pig liver, as also reported by others

[12,13]. Within a few hours of pig liver reperfusion, albumin fell to levels that are normal for pigs, but could be maintained at levels normal for baboons by the continuous intravenous infusion of human albumin [11]. Coagulation factors II (FII) (t1/2 = 65 h) and V (FV) (t1/2 = 12 h) showed porcine FII and FV production by days 3 and 1, respectively. Although baboon pre-TX antithrombin levels were significantly higher than pig levels, post-TX levels fell to normal pig levels in all measured samples except one (B7808) [11].

In the present study, we examined the kinetics of activation of graft ECs and exposure of functional TF on recipient platelets and PBMCs, from the same set of animals [10,11].

Materials and methods

Pig-to-baboon liver xenotransplantation

Baboons (*Papio anubis*, $n = 11$; Oklahoma University Health Sciences Center, Oklahoma City, OK) underwent orthotopic pig liver TX; details of surgical technique and outcome have been reported previously [10]. Four baboons with survival of less than 24 h (from technical complication or primary graft failure) were excluded from the study; seven baboons were studied in detail (Table 1). One baboon received a graft from a WT pig without immunosuppressive therapy; the liver underwent HAR and the baboon was electively euthanized 5 h after liver

Table 1. Sources of pig liver grafts, immunosuppressive protocols, and recipient survival (in days) in pig-to-baboon liver xenotransplantation.

Baboon	Graft types	Immunosuppressive therapy*	Survival
B16907	WT	–	<1
B3108	GTKO	+	6
B3208	GTKO/CD46 (+/–)	+	4
B7708	GTKO/CD46 (+/+)	+	7
B7808	GTKO/CD46 (+/–)	+	6
B18508	GTKO/CD46 (+/–)	+†	5
B18908	GTKO/CD46 (+/–)	+‡	6

The immunosuppressive protocol consisted of induction with thymoglobulin (5–10 mg/kg i.v.) and maintenance with tacrolimus (0.05–0.1 mg/kg × 2/day i.m.) mycophenolate mofetil (110 mg/kg/day i.v.), and methylprednisolone (10 mg/kg/day i.v. with slow taper). Cyclophosphamide (20 and 40 mg/kg on days −2 and −1, respectively) replaced thymoglobulin in one baboon (B18908). Cobra venom factor (3 mg/kg on days −1, 0, and 1) was added to the regimen in B18508.
(+/–) = GTKO pig heterozygous for CD46.
(+/+) = GTKO pig homozygous for CD46.
*Immunosuppressive therapy included thymoglobulin, tacrolimus, mycophenolate mofetil and methylprednisolone.
†Cobra venom factor therapy was added for 3 days.
‡Cyclophosphamide replaced thymoglobulin.

reperfusion. Six immunosuppressed baboons received grafts from a GTKO pig ($n = 1$) or from GTKO pigs transgenic for the human complement-regulatory protein, CD46 (GTKO/CD46, $n = 5$). All pigs were provided by Revivicor Inc. (Blacksburg, VA, USA).

All animal care was in accordance 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 (IACUC# 0706493).

Immunosuppressive regimen

Details are given in Table 1.

Preparation of platelets and PBMCs

Blood was collected from baboons into tubes containing ethylenediaminetetraacetic acid (EDTA, 5 mM) or acid-citrate-dextrose (ACD). After centrifugation (10 min at 150 g), the top two thirds of platelet-rich plasma were removed and centrifuged (8 min at 1400 g). The pellet was washed with buffer [137 mM NaCl, 5.3 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 4.1 mM $NaHCO_3$, and 5.5 mM glucose (pH 6.5)] containing prostaglandin E_1 (PGE_1 120 nM; Sigma, St Louis, MO, USA). Platelets were centrifuged (5 min at 150 g) to remove residual leukocytes. Platelets were maintained at 4 °C throughout the period after blood draw to avoid activation. Baboon PBMCs were isolated by a standard Ficoll-Paque density gradient, as previously described [14].

Measurement of thrombin-antithrombin (TAT) complexes

The TAT complexes were measured using a manual sandwich ELISA, as previously described [15]. Briefly, the TAT present in the sample binds to thrombin, forming a stable complex. In a second reaction, conjugated antibodies to anti-thrombin bind to free anti-thrombin determinants. The quantity of bound TAT in plasma was measured photometrically.

Flow cytometry to detect TF antigen and microparticles

Baboon platelets and PBMCs were incubated with polyclonal sheep anti-human TF (Affinity Biologicals, Ancaster, ON, Canada) or control sheep IgG (Affinity Biologicals) antibodies for 30 min. After washing, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated IgG for an additional 30 min. The samples were washed twice, and the cells resuspended with FACS buffer. Data acquisition was performed using a BD™ LSR II flow cytometer (Becton Dickinson, San Diego, CA, USA).

For microparticles, platelet-rich plasma samples were quickly thawed and centrifuged at 13000 g for 2 min at room temperature. Plasma (20 µl) was incubated with anti-human CD31 (FITC) (clone WM-59; eBioscience, San Diego, CA, USA), anti-rat CD31 (clone TLD-3A12; BD, San Jose, CA, USA) which cross-reacts only with pig CD31 but not with human CD31, anti-human CD41 (PE) (clone HIP8; eBioscience), and anti-human CD142 (TF) (FITC) (clone MATF; Affinity Biologicals) for 30 min at room temperature in the dark. Sheath buffer (500 µl) was then added and the diluted sample was subjected to flow cytometry analysis. Forward (FSC) and side (SSC) scatter thresholds were set using 0.5 and 1 µm beads, to eliminate events derived from background noise. The gate for each color was set to count the signal between 0.5 and 1 µm above the level obtained with the isotype control-treated plasma (Fig. 5a).

Recalcified clotting assay

Functional TF activity was determined by a recalcified clotting assay, as previously described [16]. Baboon platelets (2×10^6) or PBMCs (1×10^5) were suspended in 50 µl Tris-buffered saline and mixed with 100 µl of Factor VII (FVII)-deficient human plasma (Haematologic Technologies, Essex Junction, VT, USA) in glass tubes (Corning, Corning, NY, USA). A volume of 100 µl of 25 mM $CaCl_2$ in Tris-buffered saline was 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. The procedure was repeated with the addition of FVII (0.2 U/ml) (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, USA) and expressed as a procoagulant activity equivalent to nanograms (ng) of human TF.

Quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from excised grafts using Trizol (Life Technologies, Grand Island, NY, USA). Briefly, total RNA pellets were suspended in RNase-free water, followed by treatment with DNase I (Life Technologies,

Rockville, MD, USA). RNA (3 µg) from each sample was used for reverse transcription with an oligo dT (Life Technologies) and Superscript III (Life Technologies). The polymerase chain reaction (PCR) mixture was prepared using SYBR Green PCR Master Mix (PE Applied Biosystems, Foster City, CA, USA). Primers were as follows:-

Pig von Willebrand factor (vWF): 5-GGATCCGGCCTGC GCGGAACCGGTGCC-3 (forward) and 5-AGAATTCGA CTTGGGCCACTAGGGGG-3 (reverse);

Pig CD106: 5-AAGCTGAGGGATGGGAATCT-3 (forward) and 5-CAGCCTGGTTAATCCCTTCA-3 (reverse);

Pig β-actin: 5-CTCGATCATGAAGTGCGACTG-3 (forward) and 5- GTGATCTCCTTCTGCATCCTGTC-3 (reverse)

Thermal cycling conditions were 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 sec, and 60 °C for 1 min on an ABI PRISM 7000 Sequence Detection System (PE Applied Biosystems).

CH50 assay

The CH50 assay (DiaMedix, Miami, FL, USA) was used for determination of the classical pathway of complement activity in the fluid phase. Sensitized sheep erythrocytes were equilibrated at room temperature for 1 h and resuspended with a vortex or by shaking vigorously. Serum samples (5 µl) together with reference sera were added to the tubes containing sensitized sheep erythrocytes. After 1 h incubation at room temperature, the contents of the tubes were mixed by inverting them 3–4 times. After centrifugation for 10 min at 1750 *g*, hemolysis was determined in the supernatant by measuring the absorbance of released hemoglobin at 412 nm compared to the references.

Immunofluorescence studies

Cryostat sections of the pig liver xenografts were fixed in acetone and incubated with the following primary antibodies overnight – mouse anti-porcine P-selectin (clone 12C5) and CD106 (10.2C6) (generous gifts from Professor D.O. Haskard, Imperial College London, UK); custom rabbit anti-porcine TF raised against a synthetic peptide comprising the sequence IMRNVKETYV present in the porcine TF protein (NCBI reference sequence NP_998950.1); mouse anti-porcine E-selectin (clone 1.2B6; Sigma); mouse anti-human vWF (clone F8/86; DAKO, Carpinteria, CA, USA); mouse anti-primate CD45 (clone 5H9; BD); mouse anti-human CD42a (clone fmc25; AbDSerotec, Raleigh, NC, USA); sheep anti-human TF (Affinity Biologicals); sheep anti-human fibrin (clone SAFNE; Affinity Biologicals); mouse anti-porcine CD31 (clone APG311; Antigenix America, Huntington

Station, NY, USA) [17,18]; anti-human CD41 (clone ab63983; Abcam, Cambridge, MA, USA); rabbit anti-human IgG (DAKO), rabbit anti-human IgM (DAKO); rabbit anti-human C3 (DAKO); mouse anti-human C5-9 (DAKO); mouse anti-human CD68 (DAKO); mouse anti-human CD20 (DAKO); rabbit anti-human CD3 (DAKO). After washing, the sections were incubated with appropriate secondary antibodies for 1 h [CyChrome 2 anti-sheep IgG, CyChrome 3 anti-mouse IgG, CyChrome 5 anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA)]. Nuclei were stained with DAPI (4,6-diamidino-2-phenylindole; Molecular Probes, Eugene, OR, USA). After paraformaldehyde-fixation, the tissues were prepared with poly-L-lysine-coated slides. Images were viewed through a Nikon E-800 microscope (Melville City, NY, USA).

Electron microscopy

Liver tissue was fixed with 2.5% glutaraldehyde in PBS. Transmission electron microscopy was performed, as previously described [19].

Statistical analysis

Data are presented as mean ± 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

Development of CC after pig liver xenotransplantation

The WT pig liver graft in the non immunosuppressed baboon underwent HAR; the baboon developed severe thrombocytopenia and was euthanized 5 h after reperfusion. All six baboons with genetically engineered pig liver grafts developed CC and either died or were euthanized after 4–7 days (median 6 days) (Table 1). CC presented as profound thrombocytopenia and thrombin formation within the first hour in five recipients and within 24 h in the sixth baboon.

One baboon (B3208) did not develop quite so profound thrombocytopenia within 24 h. The reason remains uncertain. This recipient had very high blood levels of tacrolimus (>50 ng/ml) on days 1–2 (despite being administered the same dose of tacrolimus as the other baboons), which may possibly have been a factor. (In subsequent experiments, we controlled the tacrolimus level by omitting the drug after TX until there was evidence of good hepatic function) [10].

In baboons in which CC developed within 2 h, platelet counts fell from 270 ± 60 to $50 \pm 20 \times 10^3/\mu l$ (Fig. 1a) and continued to decrease subsequently. D-dimer
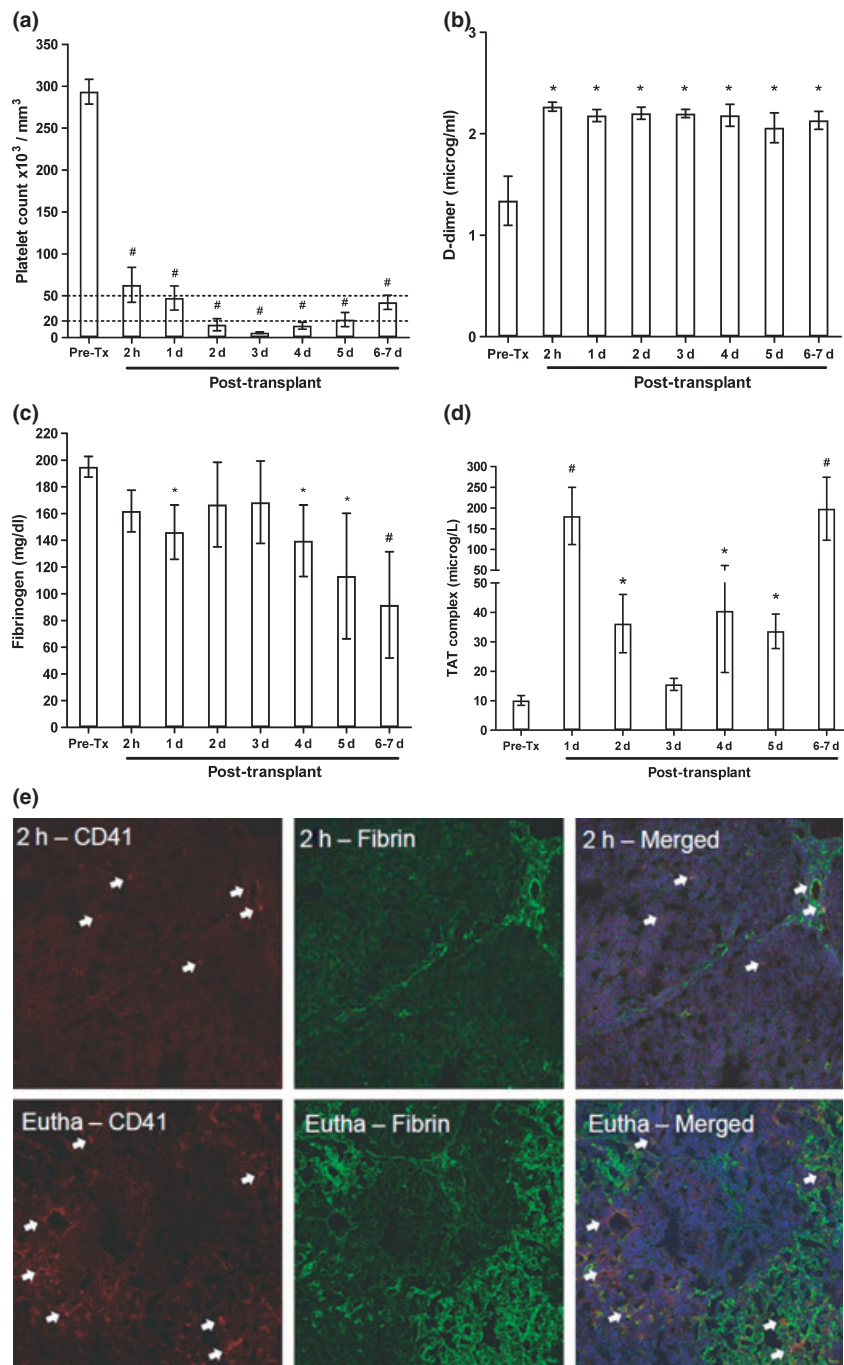
Figure 1 Development of thrombocytopenia and thrombin formation after pig-to-baboon liver xenotransplantation ($n = 6$). (a) Platelet count, (b) plasma D-dimer, (c) fibrinogen, and (d) thrombin-antithrombin complexes before pig liver transplantation (TX), 2 h after TX, and at euthanasia (1–7 days) in baboons ($*P < 0.05$, $\#P < 0.01$ vs. pre-TX) (e) Immunofluorescence staining (200×) showed fibrin deposition (green), platelets (red), and cell nuclei (blue) at 2 h and at euthanasia (day 6) following GTKO/CD46 pig liver TX (B7808) (Arrows indicate platelet deposition). (h = hour; d = day).

increased from 1.25 ± 0.55 to 2.12 ± 0.06 µg/ml, and remained at higher values throughout the experiment (Fig. 1b). Although there was evidence of pig fibrinogen production by the transplanted liver [11], levels of fibrinogen decreased slowly throughout the experiment (Fig. 1c). As a direct marker of thrombin formation in the peripheral blood, significantly increased TAT complexes were noted on day 1 (180 ± 154 µg/l) in comparison to

pre-TX values (10 ± 4 µg/l) ($P < 0.01$), which could possibly be explained by the effect of the surgical procedure. However, post-TX TAT complex values remained significantly higher than the pre-TX values (Fig. 1d).

Another direct marker of thrombin formation, prothrombin fragments $1 + 2$, could not be measured because the available human polyclonal kit did not detect porcine prothrombin fragments. However, fibrin formation was

documented in the graft (Fig. 1e). Electron microscopy confirmed the results of immunohistochemical staining in that, in the 2 h biopsies, there was significant fibrin deposition in the liver sinusoids together with platelet aggregation (Fig. 2).

Baboon platelets and PBMCs are activated to expose TF early after pig liver xenotransplantation

To investigate the source of TF, platelets and PBMCs were isolated from the blood. Baboon TF antigen on platelets and PBMCs was detected by flow cytometry and TF activity by the recalcified clotting assay 2 h after liver TX (Fig. 3a and b). Some platelet and PBMC aggregates were found in the grafts (Fig. 3c). These observations indicated that recipient platelets and PBMCs were activated early after reperfusion.

Minimal EC activation in the pig liver grafts

To investigate the role of graft ECs in the initiation of CC, type I and type II EC activation was examined by sequential immunofluorescent staining of the grafts. In the baboons with genetically engineered pig livers that developed CC 2 h after reperfusion, type I EC activation (P-selectin) ($n = 5$), type II EC activation (E-selectin, CD106) ($n = 4$), and TF exposure on graft ECs were not increased at 2 h (Fig. 4a and b). However, activation was present at the time of death or euthanasia of the baboon
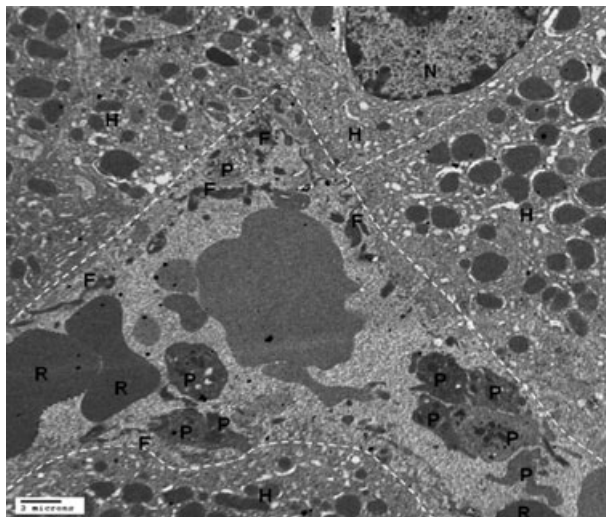
Figure 2 Electron micrograph of pig liver xenograft 2 h after reperfusion. Aggregation of platelets with fibrin deposition along the sinusoidal endothelial cells was noted. The appearance of hepatocytes was normal. Dashed white lines indicate endothelial cells lining the sinusoids. F = fibrin, H = hepatocytes, N = Nucleus, P = platelets, R = red blood cells. (Solid black bar indicates 2 μm).

(days 4–7) and in the WT pig graft that underwent HAR (B16907) (Fig. 4a and b). In contrast, von Willebrand Factor (vWF) expression was detected 2 h after reperfusion in all grafts (Fig. 4c). The expression of CD106 and vWF was consistent with mRNA expression determined by quantitative RT-PCR (Fig. 4d).

Platelet and endothelial cell microparticles

Microparticles from the five longest-surviving recipients of pig liver grafts were measured (Fig. 5). To identify whether the microparticles originated from pig liver ECs, baboon platelets, or baboon ECs, plasma samples were incubated with (i) anti-human CD41, which specifically cross-reacts with baboon platelets only, (ii) anti-human CD31, which stains baboon ECs and platelets, (iii) anti-rat CD31, which specifically binds to pig ECs (which could only originate from the pig liver graft), and (iv) anti-human TF, which cross-reacts with baboon TF.

Anti-pig CD31-staining suggested that pig liver ECs did not significantly contribute to the microparticles in the plasma (Fig. 5b). However, staining for anti-human CD31 (platelets + recipient ECs), anti-human CD41 (platelets only), and anti-human CD31$^+$/CD41$^-$ (recipients ECs only) suggested that the microparticles originated mainly from baboon platelets and baboon ECs. The main source of TF in the plasma was platelets (anti-human CD41$^+$/TF$^+$) and recipient ECs (anti-human CD31$^+$/CD41$^-$/TF$^+$) (Fig. 5b). The fact that anti-human CD41 staining did not significantly change throughout the experiment (Fig. 5b) supports our previous observations that platelets do not completely disappear from the circulation after liver xenoTX, but are not able to be counted accurately attributable to aggregation of platelets and platelets with PBMCs, particularly within the xenograft [20]. Additional evidence suggesting the continuing presence of platelets in the circulation is the relatively stable correlation of platelet count with anti-human CD41 (Fig. 5c).

The correlation between anti-human CD41 and anti-pig CD31 staining was significant, suggesting that anti-pig antibody was specific for porcine proteins and did not stain baboon platelets (Fig. 5d).

The correlation between anti-human CD31-staining and the number of platelets indicated that, when the platelet count was high (pre-TX, when platelets were not activated), the expression of CD31 (PECAM-1, platelet endothelial cell adhesion molecule-1) was low. However, when the platelet count fell after TX, which could have been attributable to (i) activation of platelets, (ii) aggregation of platelets or of platelets and PBMCs, and/or (iii) phagocytosis of platelets by pig liver ECs [12,20,21], anti-human CD31 expression significantly increased (Fig. 5c).
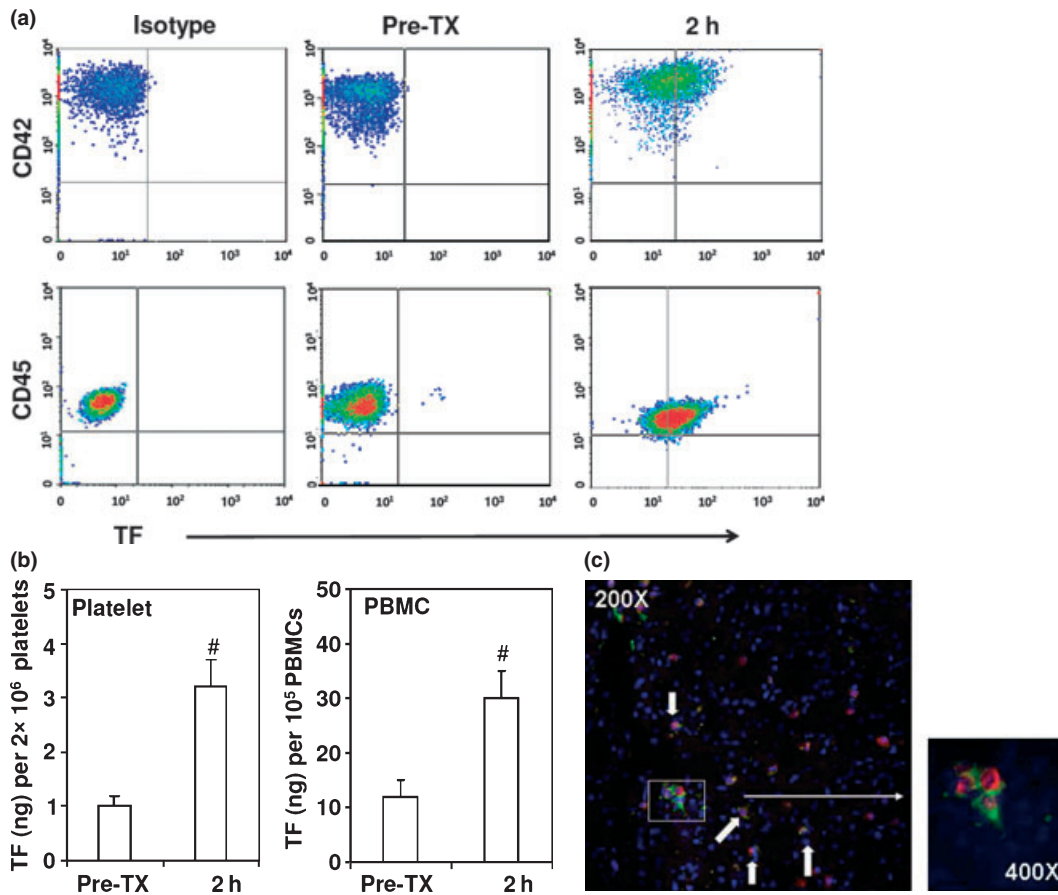
Figure 3 Recipient platelets and peripheral blood mononuclear cells (PBMCs) possess functional tissue factor (TF) and aggregate after pig-to-baboon liver xenotransplantation. (a) TF antigen on recipient platelets and PBMCs was measured using flow cytometry (CD42: platelets; CD45: PBMCs) (The figure shows animal B3208, MFI ratio: 6.32 in platelets), and (b) TF activity was measured using the recalcified clotting assay pre-transplantation (TX) and 2 h after reperfusion of the liver (2 h) (#$P < 0.01$) (c) The grafts were stained for platelets with CD41 (green) and for PBMCs with CD45 (red). Platelets and PBMCs aggregated (arrows) in the graft (B7808) 2 h after TX (Left ×200; right ×400).

Baboon humoral response to the pig liver graft

Serum anti-porcine (Gal + nonGal) and anti-nonGal IgG and IgM antibodies were measured to determine the humoral immune response. When compared with pre-TX levels, anti-porcine and anti-nonGal IgG and IgM levels remained unchanged throughout the post-TX course (Fig. 6a), indicating a lack of sensitization. The WT pig graft that underwent HAR showed severe hemorrhagic necrosis (not shown), whereas 2 h after reperfusion the genetically engineered pig liver grafts demonstrated almost normal histology (Fig. 6b). Immunofluorescent staining showed significant deposition of IgM, IgG, C3, and C5-9 in HAR, but minimal deposition of IgM and very minimal to no deposition of IgG, C3, and C5-9 in the genetically engineered pig grafts, even though thrombocytopenia had already developed (Fig. 6c), although minimal IgM deposition was seen in occasional sections

in some cases. Moreover, in B18508, when complement activity was eliminated by the administration of cobra venom factor (Fig. 6d), thrombocytopenia still developed immediately after liver TX.

Cellular infiltration in the grafts

To determine the extent of the cellular immune response, macrophage (CD68), B (CD20), and T (CD3) cellular infiltration in the grafts were evaluated by immunofluorescent staining. When HAR developed in the WT pig liver, significant numbers of B and T cells were found in the graft, with a smaller number of macrophages. In the baboons with genetically engineered pig liver grafts, there was no significant infiltration of macrophages, B or T cells 2 h after reperfusion. Macrophages, but not B and T cells, were present in the grafts by the time the baboons were euthanized (days 4–7) (Fig. 7).
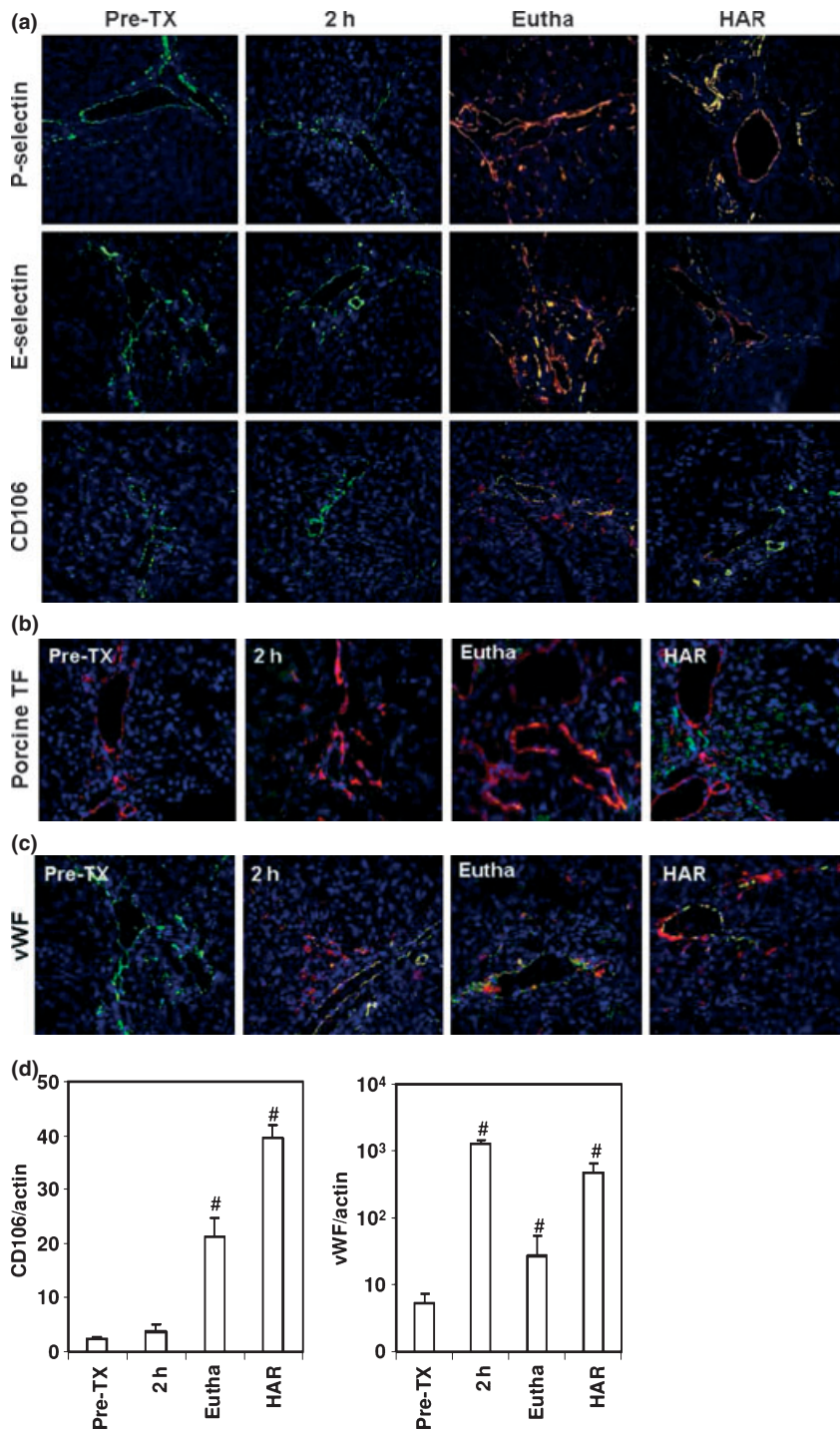
Figure 4 Minimal endothelial cell (EC) activation in pig liver grafts during onset of consumptive coagulopathy. Genetically engineered pig liver grafts were examined pre-transplantation (TX), 2 h after reperfusion, and at the time of death or euthanasia (days 4–7). The wild-type pig liver graft that had undergone hyperacute rejection (HAR) was excised at 5 h. Immunofluorescent staining (200×) showed minimal expression of (a) P-selectin (red), E-selectin (red), CD106 (red) on the ECs (CD31, stained in green) or of (b) tissue factor (green) (CD31, red) 2 h after reperfusion, compared with expression at euthanasia (eutha) or after HAR. In contrast, (c) von Willebrand Factor (vWF) (red) (CD31, green) was already expressed in the grafts 2 h after perfusion. (d) The expression of CD106 and vWF mRNA was consistent with the findings in b and c ($^{\#}P < 0.01$, compared with pre-TX).
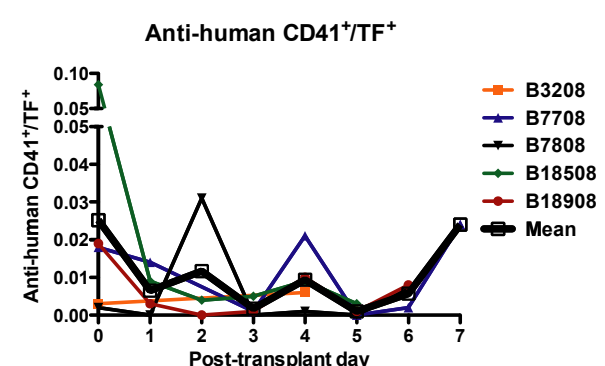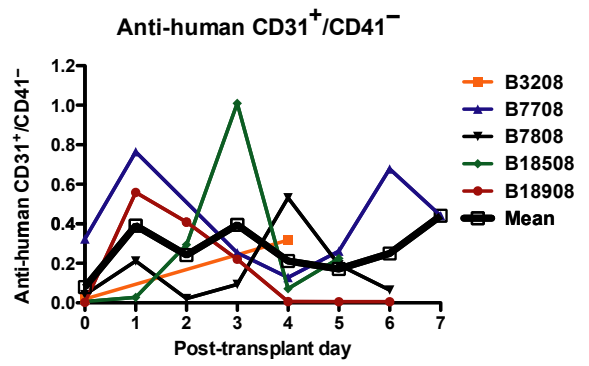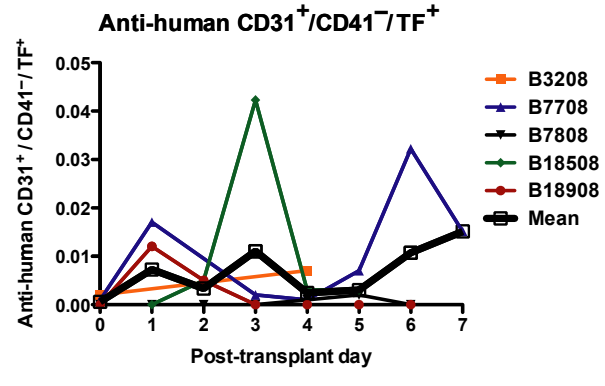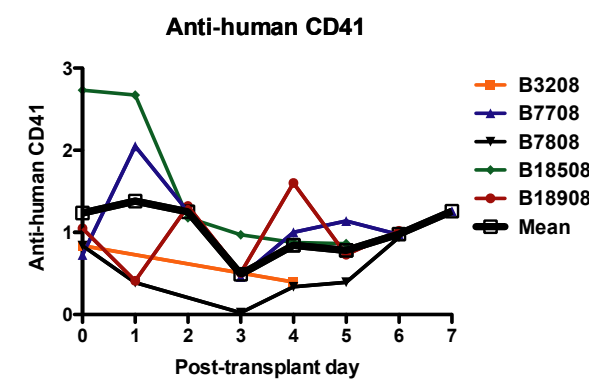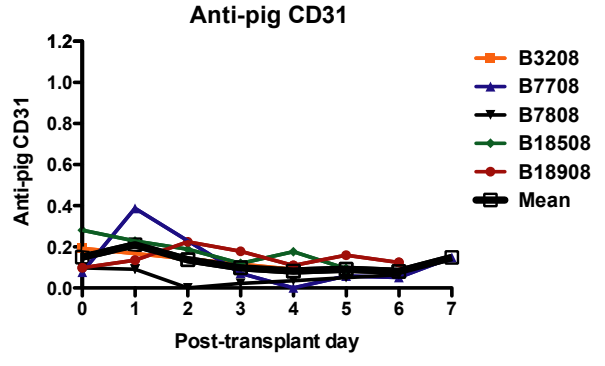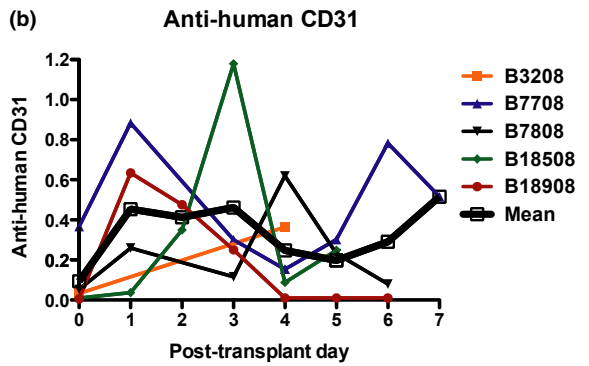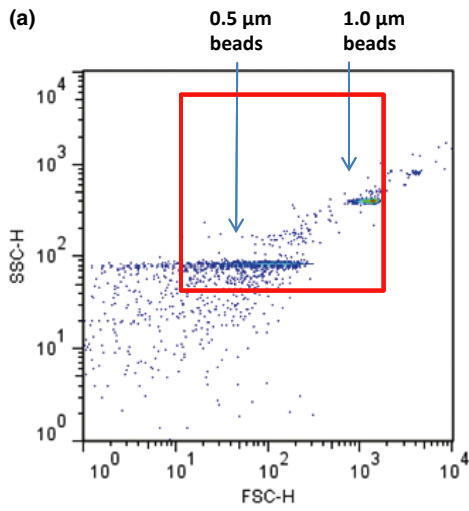
Discussion

In a pig-to-baboon kidney TX model [22], we observed that TF exposure on recipient platelets occurred earlier than on leukocytes and was associated with the development of thrombocytopenia, which we suggested was the first feature of CC. There were minimal features of an immune response at this time (no P- or E-selectin or TF expression on ECs, no cellular infiltration, no or minimal immunoglobulin or complement deposition).

Importantly, the histopathology of the excised grafts at 4–7 days in most baboons with developing CC continued to show a negligible immune response (with no deposition of IgG or C3, and no infiltration with
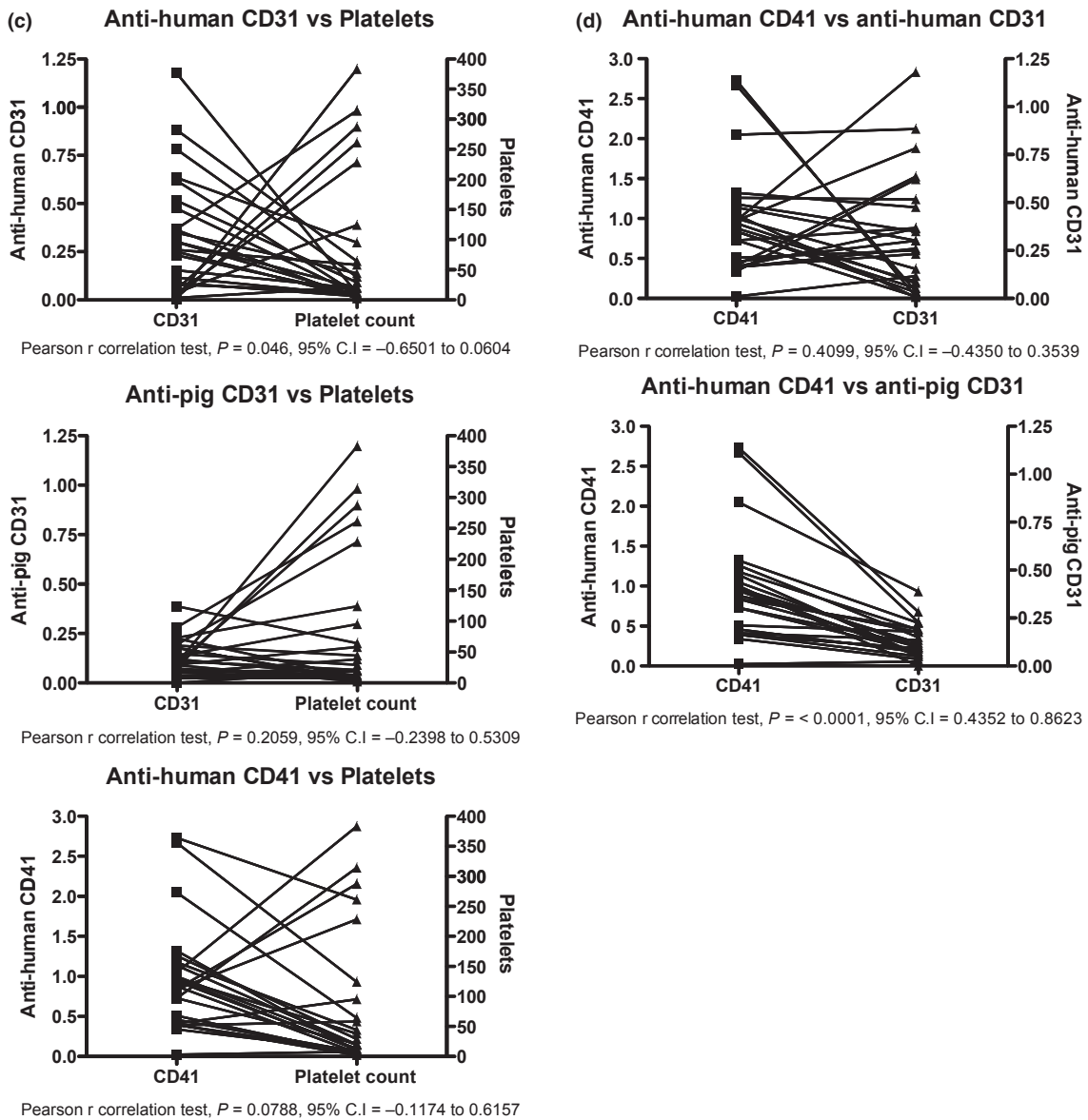
(a)

(b)

Anti-human CD31

Anti-pig CD31

Anti-human CD41

Anti-human CD31$^+$/CD41$^-$/TF$^+$

Anti-human CD31$^+$/CD41$^-$

Anti-human CD41$^+$/TF$^+$

(c)

Anti-human CD31 vs Platelets

Pearson r correlation test, *P* = 0.046, 95% C.I = –0.6501 to 0.0604

Anti-pig CD31 vs Platelets

Pearson r correlation test, *P* = 0.2059, 95% C.I = –0.2398 to 0.5309

Anti-human CD41 vs Platelets

Pearson r correlation test, *P* = 0.0788, 95% C.I = –0.1174 to 0.6157

(d)

Anti-human CD41 vs anti-human CD31

Pearson r correlation test, *P* = 0.4099, 95% C.I = –0.4350 to 0.3539

Anti-human CD41 vs anti-pig CD31

Pearson r correlation test, *P* = < 0.0001, 95% C.I = 0.4352 to 0.8623

Figure 5 continued

macrophages, B and T cells) unlike typical AHXR. These observations suggested that activation of platelets and the initiation of CC would appear *not* to result

from immune-mediated mechanisms [22]. Nevertheless, it is difficult to exclude a role for the remaining cells (e.g., lymphocytes, macrophages) after depletion with

Figure 5 Measurement and comparison of platelet and endothelial cell microparticles. (a) Identification of microparticles by flow cytometry with FSC and SSC. Red box area indicates 0.5 μm and 1.0 μm size microparticles. (b) Anti-human CD31 [platelets + recipient endothelial cells (ECs)], and anti-human CD31$^+$/CD41$^-$ (recipient ECs only) increased after pig liver xenoTX. Anti-pig CD31 (donor pig liver ECs only) activity remained stable and low pre- and post-transplantation (TX). Anti-human CD41 (platelets only) remained mainly stable throughout the experiment. Tissue factor (TF) staining on platelets only (anti-human CD41$^+$/TF$^+$) and recipient ECs only (anti-human CD31$^+$/CD41$^-$/TF$^+$) suggested that the source of TF could be from both. All ''0'' time-points indicate pre-TX levels. (c) Correlation of platelet count with anti-human CD31, anti-pig CD31, and anti-human CD41. Activation of platelets after TX decreased platelet count and increased the expression of human CD31 significantly. However, normal platelet count or thrombocytopenia did not change the expression of CD41 on platelet microparticles. (d) Correlation of anti-human CD41 with anti-human CD31 and anti-pig CD31. Very significant correlation with anti-pig CD31 indicated anti-pig antibody did not cross-react with baboon platelets (see also Fig. 5c for the correlation of platelet count with anti-pig CD31).

anti-thymocyte globulin. The few remaining cells might initiate CC. In a pig-to-baboon cardiac TX model, evidence by Byrne *et al.* [23] suggests that increased immunosuppression, rather than increased anticoagulation, extends cardiac xenograft survival by delaying the development of thrombotic microangiopathy and the onset of coagulation dysfunction.

Our observations in the current pig-to-baboon liver TX model indicated that there was no macrophage activation in the liver tissues (2 h vs. euthanasia), but there was increased neutrophil infiltration at euthanasia in comparison to 2 h biopsies [24]. Moreover, in two experiments in which we depleted macrophages in the donor pig with clodronate liposomes [10], thrombocytopenia occurred in the baboon with the same severity after liver TX (although there was primary nonfunction of the transplanted liver) [24].

Profound thrombocytopenia developed immediately after reperfusion, not only in the baboon in which the WT pig liver graft underwent HAR, but also in all recipients of genetically engineered pig livers. In biopsies taken 2 h after reperfusion, TF was detected on recipient platelets and PBMCs, with early formation of fibrin. Electron microscopy biopsies demonstrated significant fibrin deposition in the liver sinusoids together with platelet aggregation as early as 2 h after graft reperfusion. The early changes in D-dimer, fibrinogen, and TAT could have been related to the surgical procedure [25]. There was evidence of a biphasic response in that TAT showed an immediate rise, followed by a decline, and then another rise. Fibrinogen showed an initial reduction, then a rise, and subsequent fall. These changes suggested an initial effect of the surgical procedure, followed by a transient return toward normal, followed by a distinctive change associated with the presence of the xenograft. There were also subsequent changes compatible with CC.

Although CC was initiated early after reperfusion, the grafts remained functioning (for up to 7 days post-TX) and the histopathologic findings revealed extensive areas of normal liver structure (quite unlike the features seen in HAR or AHXR) [24]. Normalization of liver function tests and synthesis of proteins (complement and coagulation factors) were documented in the recipients that survived >24 h (*n* = 6) [10,11].

A critical question is whether the mechanisms of platelet activation were dependent or independent of the immune response. It has generally been believed that activation of platelets during AHXR is secondary to activation of ECs in the graft following binding of xenoreactive antibodies and complement activation [2,3]. Therefore, the reasoning is that the TX of organs from pigs that over-express a complement-regulatory protein (e.g., CD46 or CD55) combined with an intensive immunosuppres-

sive regimen or a tolerance-inducing regimen might be expected to overcome the problems associated with AHXR.

In xenotransplantation, AHXR has been considered to be associated with type II activation of ECs [26], although the mechanism still remains uncertain. Primate serum induces type II activation of PAECs (up-regulation of selectins or VCAM), but is dependent on the presence of complement [27]. This type of activation is associated with the binding of the IgG3 subclass of anti-Gal antibodies [28] or of anti-nonGal antibodies, although these make less of a contribution [29]. However, direct targeting of Gal epitopes by an agonist can evoke type II EC activation in the absence of complement [30]. Other studies demonstrated induction of IL-8 and plasminogen activator inhibitor-1 in PAECs after activation with xenoreactive antibodies without the involvement of complement [31].

In the present study, molecules of EC type I or II activation (e.g., P-selectin, E-selectin, TF) were not detected on the graft ECs 2 h after reperfusion, but were positive in the grafts at the time of baboon death or euthanasia. Measurement of microparticles showed that staining with anti-pig CD31, which specifically binds to pig liver ECs, did not significantly change throughout the experiment, suggesting minimum release of microparticles from pig liver ECs. At the same time-points, the deposition of IgG, C3, and C5-9 was largely or completely absent, although there was minimal IgM deposition in some cases [24]. In addition, the baboons did not become sensitized (by the evidence of unchanged levels of serum anti-porcine and anti-nonGal antibodies) even though this would perhaps not be anticipated to occur in the 4–7 days of baboon follow-up. Importantly, CC still developed in the baboon (B18508) in which complement activity had been eliminated by the administration of cobra venom factor. Hence, despite the slower development of thrombocytopenia in the single baboon with high tacrolimus levels (which may have been associated with a toxic effect), we suggest that thrombocytopenia and CC were probably not initiated by type I or II EC activation.

Whether type II activation of ECs is attributable to factors other than antibodies or complement remains under investigation. It is speculated that platelets activated by retracted PAECs secrete chemokines to recruit and activate host monocytes and NK cells [31–35]. The latter, when activated, secrete additional cytokines (e.g., TNF-α, interleukin-1, and interferon-γ), which then provide a stimulus for EC activation, with consequent coagulation and inflammation.

We found that porcine vWF was highly expressed on graft ECs during the development of CC. This observation was consistent with previous reports [4,36,37]. Porcine vWF on ECs has been demonstrated to activate
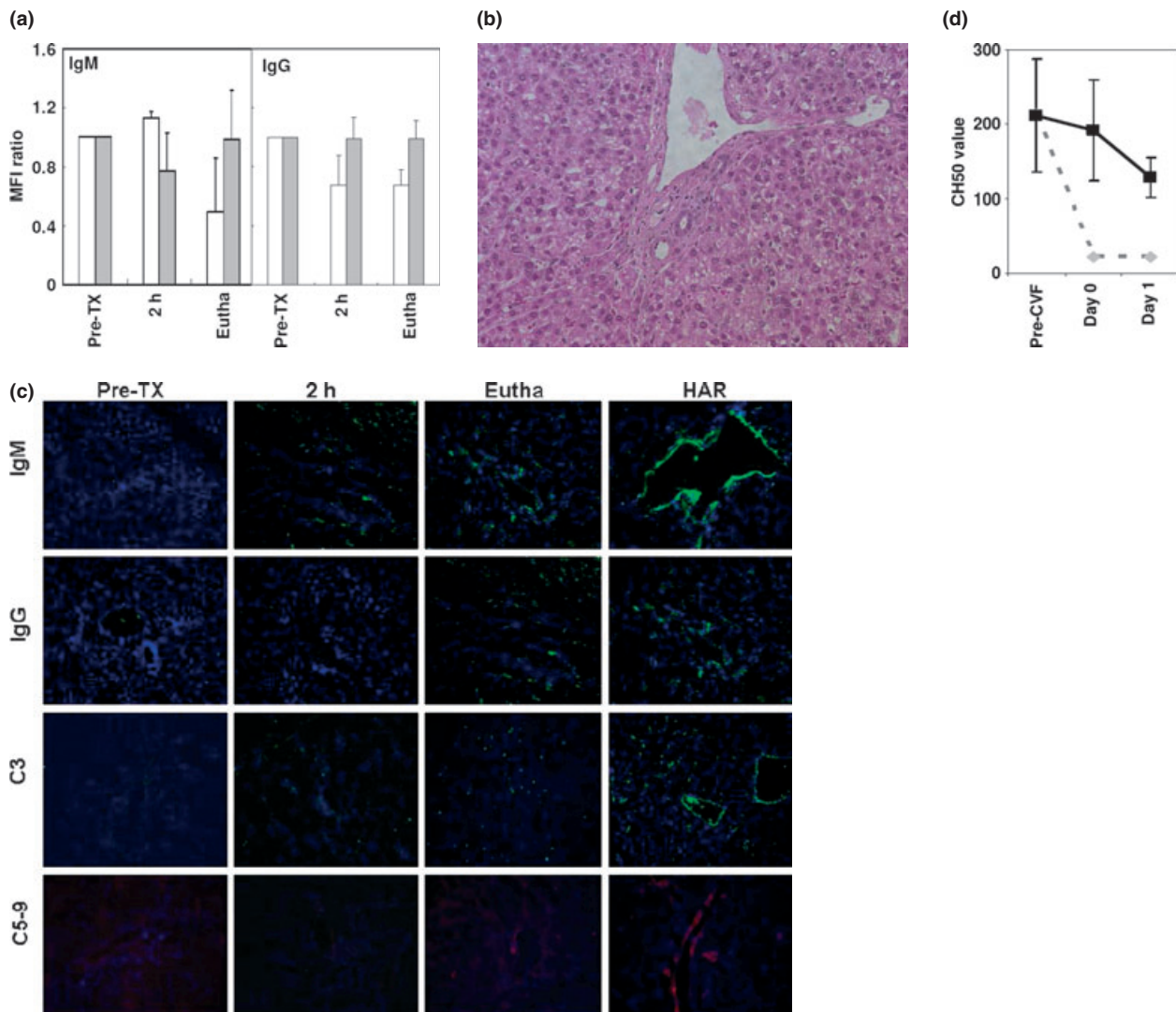
Figure 6 Minimal antibody and complement deposition in the grafts at the time of onset of consumptive coagulopathy. (a) Genetically engineered pig liver grafts were examined pre-TX, 2 h after reperfusion, and at the time of death or euthanasia (days 4–7). The wild-type (WT) pig liver graft that had undergone hyperacute rejection (HAR) was excised at 5 h. Ratio of mean fluorescence intensity (MFI) of serum anti-porcine (open bars) and anti-nonGal (gray bars) IgM and IgG levels pre-transplantation (TX) (day -1), 2 h after reperfusion, and at euthanasia (measured using 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. There were no statistical differences between levels at any of the time-points. Antibody measurement and the identification of anti-nonGal and anti-porcine antibodies were performed using cells from GTKO and WT pigs, respectively, as previously described by our group [14]. (b) Histology of graft in B7808 2 h after reperfusion showed normal structure without hemorrhage and/or necrosis. (c) The deposition of IgM (green), IgG (green), C3 (green), and C5-9 (red) was absent or minimal 2 h after reperfusion and at euthanasia. In contrast, there was significant deposition in the graft that underwent HAR. (d) Serum complement activity was eliminated after the administration of cobra venom factor (broken line) (B18508), compared with 5 CVF-untreated baboons (solid line).

primate platelets (without the requirement of shearing force) through an aberrant A1 domain [36,37]. Pigs that were deficient in vWF provided modest survival benefit in a lung xenotransplantation model [38]. Recent *in vitro* studies in our laboratory demonstrate that blocking vWF expression on pig ECs by antibody could prevent the activation of primate platelets induced by porcine ECs with-

out the need for the presence of antibody and/or complement [9]. vWF plays a critical role in the early stage of hemostasis by promoting the adherence of platelets to subendothelium [39]. Moreover, recent studies recognized that severe vWF/ADAMTS13 imbalance during the anhepatic phase of orthotopic liver TX [40,41] could aggravate the accumulation of vWF and subsequent plate-
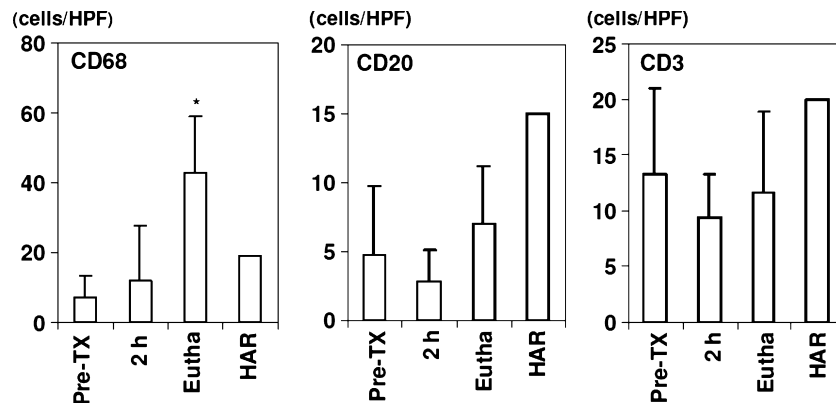
Figure 7 Cellular infiltration in the grafts during the onset of consumptive coagulopathy. Genetically engineered pig liver grafts were examined pre-transplantation, 2 h after reperfusion, and at the time of death or euthanasia (days 4–7). The wild-type (WT) pig liver graft that had undergone hyperacute rejection (HAR) was excised at 5 h. The number of cells infiltrating the graft was counted under the microscope (200×), and are indicated per high-power field. In those baboons that developed CC ($n = 6$), there was no significant infiltration of macrophages (CD68), B cells (CD20), or T cells (CD3) 2 h after reperfusion. The number of macrophages, but not B or T cells, had increased by the time the baboon was euthanized (*$P < 0.05$). In the WT pig graft that underwent HAR, the number of infiltrating macrophages, B and T cells was increased within hours.

let activation. This may provide a plausible explanation why CC is initiated after liver xenotransplantation.

The Indianapolis [12] and Boston [21] groups have recently provided evidence to suggest that platelets are rapidly phagocytosed by hepatic sinusoidal endothelial cells. In our electron micrographs of the 2 h biopsies of the pig livers, we were unable to determine platelet phagocytosis, although platelet aggregation with fibrin deposition was clear. Whether platelets are lost through aggregation or phagocytosis, however, the initial factors contributing to platelet activation may be the same. Moreover, our measurement of microparticles suggested that their origin was mainly from platelets and recipient ECs in the plasma.

In summary, although there may be several factors influencing the development of thrombocytopenia after liver TX [42–44], activation of platelets and severe thrombocytopenia remain a major hurdle for successful pig-to-primate liver xenoTX. We provide some evidence suggesting that thrombocytopenia and CC is not initiated by activation of graft endothelium in response to the immune response, but that activation of recipient platelets occurs after exposure of the platelets to graft ECs. However, the weaknesses in our argument are (i) the early D-dimer and TAT data could be attributable to the effect of surgery, and (ii) the fibrinogen data show an acute phase rise (with eventual fall) but do not provide absolute support for CC being the cause of the immediate thrombocytopenia. Our recent observations suggest that the 'thrombocytopenia' may be associated with falsely low platelet counts owing to the abovementioned factors

[20,24]. Whether or not minimal TF expression on graft ECs is an initiating factor in the development of thrombocytopenia therefore remains inconclusive. The exact factors responsible for the effect of pig ECs on primate platelets require additional investigation.

Additional understanding of the interaction between porcine ECs and primate platelets should be sought as this may allow genetic modification of the organ-source pig or the development of a successful therapeutic approach. Additional suppression of the immune response (with the concomitant risks of infection or other complications) is not likely to resolve the problem of CC completely.

Authorship

CCL: co-designed the study and experiments, performed immunohistological and *in vitro* studies, participated in the surgical procedures, co-wrote the manuscript. BE: performed surgical procedures, animal care, follow-up, *in vivo* procedures and *in vitro* assays, and co-wrote the manuscript. CL, GJE, HH, ME: assisted with surgeries, animal care, follow-up, and performed *in vitro* assays. VYB: provided important materials to the study, participated in discussions. DBS: performed electron microscopic studies. KE, SCR: measurement and interpretation of microparticles. DA: supervised the production of genetically engineered pigs. AD: provided important input into the study, and participated in final discussions. DKCC: co-designed the study and experiments, participated in the surgical procedures, co-wrote the manuscript. BG: co-designed the study and experiments,

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