ORIGINAL ARTICLE

Investigation of the influence of xenoreactive antibodies on activation of complement and coagulation in an *ex vivo* perfusion animal study using porcine kidneys

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SUMMARY

During pig-to-primate xenotransplantation or perfusion of porcine organs with human blood, a xenogeneic coagulopathy with consecutive development of thrombotic microangiopathy (TMA) can be observed. The aim of this study was to elucidate the influence of the reduction of xenoreactive natural antibodies on the coagulopathy using an *ex vivo* perfusion system. Thirteen perfusion experiments using landrace wild-type porcine kidneys were performed in three different experimental groups: *autologous*, *xenogeneic*, and *immunoadsorption*. During and after perfusion, blood and tissue samples were collected to assess markers of coagulation, complement, inflammation, and endothelial activation. Immunoadsorption prior to perfusion did not prolong perfusion time (174 min ±28) compared to xenogeneic (182 min ±22) experiments, whereas autologous perfusion was possible for maximum of 240 min in all experiments. Activation of coagulation was similar comparing perfusions after immunoadsorption (D-Dimer 24 186 µg/l ±5813; TAT 566 µg/l ±34) to xenogeneic (D-Dimer 22 175 µg/l ±7826, TAT 600 µg/l ±0) experiments. But antibody-mediated complement activation was reduced in the *immunoadsorption* group. TNF-alpha and markers of endothelial cell activation were lower in the *immunoadsorption* group compared to the xenogeneic experiments. In this *ex vivo* perfusion model, we observed that marked removal of xenogeneic antibodies can reduce complement activation via the classical pathway as well as endothelial cell activation and inflammation. Immunoadsorption cannot prevent the activation of the terminal complement cascade and coagulation.

Transplant International 2019; 32: 546–556

Key words
coagulation, *ex vivo* perfusion, porcine kidneys, xenotransplantation

Received: 29 April 2018; Revision requested: 10 June 2018; Accepted: 23 December 2018;
Published online: 22 January 2019

Introduction

Xenotransplantation is a promising approach to overcome the shortage of donor organs for allotransplantation for patients with end-stage organ diseases. Pigs are considered to be the most suitable xenograft source because of similarities in organ size and physiology to humans [1]. However, immunological and molecular incompatibilities

doi:10.1111/tri.13396

between pigs and humans represent a hurdle to xenotransplantation [1,2].

After pig-to-primate xenotransplantation or perfusion of porcine organs with human blood, a hyperacute rejection (HAR) driven by preformed xenoreactive natural antibodies (XNA) can occur immediately within minutes to hours after the transplantation [3]. With a later onset (days to months), the acute humoral xenograft rejection (AHXR) takes place, based on preformed and induced XNA [4].

These antibodies can be divided into anti-Gal and anti-non-Gal, depending on the binding epitope on the porcine endothelial surface. While anti-Gal-antibodies bind to a specific epitope, the galactose-α-1,3-galactose (Gal), produced by the enzyme α1,3-galactosyltransferase (GalT) on the endothelium, non-Gal-antibodies bind to a variety of epitopes on the endothelium [5]. Consequently, endothelial cells become activated and transition from an anticoagulant to a procoagulant state. Additionally, the binding of antibodies also activates the recipient's complement system [6,7].

Hyperacute rejection was successfully addressed by the development of GalT knockout (GalT-KO) pigs. However, AHXR, as a combination of cellular and humoral processes, can still occur [1–4]. It can be induced by preformed anti-non-Gal antibodies or elicited XNA and is associated with complement activation, endothelial cell activation, and xenogeneic coagulopathy leading to thrombotic microangiopathy (TMA) [6–12]. This xenogeneic rejection results in a failure of the transplanted organs or even death of the recipient [9,11,12].

Several approaches have been made to control complement activation either by depletion of anti-Gal antibodies, by application of complement regulators, or by using pigs transgenic for human complement regulatory proteins [12–15]. These approaches lead to prolonged organ survival, but organs are still rejected after lasting for days, weeks, or months [15,16].

The xenogeneic coagulopathy is characterized by activation of the recipient coagulation (e.g., D-Dimer) and the occurrence of microthrombi in the xenograft. Contributing factors are endothelial cell activation, circulating tissue factor, and impaired regulation of coagulation [7,12,17,18]. Although coagulopathy is often associated with xenogeneic rejection, this phenomenon was also observed in some animal models without evidence for ongoing xenograft rejection [19].

We have previously shown that high concentrations of anticoagulant proteins (activated protein C [APC] [17], or antithrombin [AT] [12]), or suppression of procoagulant mechanisms (tissue factor knock down [TFKD] [20]) can reduce the activation of coagulation in an *ex vivo* perfusion model. Furthermore, inhibition of the terminal complement cascade reduced the activation of coagulation in a flow chamber assay [21].

Of note, it has never been clarified whether xenogeneic activation of complement and coagulation was just consequence of endothelial damage by xenoreactive antibodies, or whether they were provoked, at least to some extent, by independent mechanisms. Molecular incompatibilities suggest that the host's coagulation system cannot be properly controlled by the donor organ's anticoagulant proteins [22,23]. The same might be true for complement control on the donor organ's endothelial surface as suggested by the beneficial effects of transgenic expression of human complement regulators [24].

In this study, we addressed the question of xenogeneneic coagulopathy in an *ex vivo* organ perfusion model by depleting xenoreactive antibodies and studying how that reduced the activation of complement and coagulation. Our results suggest that xenoreactive antibodies are not the only reason for xenogeneic activation of complement and coagulation.

Materials and methods

Experimental protocol

Thirteen *ex vivo* kidney perfusions were performed in three different groups (*autologous*, *xenogeneic*, and *immunoadsorption*). In the *autologous* group, three porcine wild-type kidneys were perfused with porcine blood from the same kidney donor animal, serving as negative control experiment in order to exclude that activation of coagulation was mediated by experimental procedure (e.g., by perfusion of the circuit). In the *xenogeneic* group, five porcine wild-type kidneys were perfused with human blood (type AB positive), serving as positive control experiment in order to determine the aberrant activation of coagulation within the xenogeneic setting. In the intervention group *immunoadsorption*, five porcine wild-type kidneys were perfused with human blood (type AB positive) after immunoadsorption and subsequent reduction of IgM and IgG antibodies.

Kidney recovery

Kidney recovery was performed according to our established experimental protocol [17].

Pigs were operated at an age of 3 months, weighing between 25 and 35 kg (Ferkel-Erzeuger-Gemeinschaft

Springe, Springe, Germany). Pigs were maintained in the animal housing facility at the Hannover Medical School, and studies were performed in accordance with the German Animal Welfare Act regulating animal experiments. The regulatory authority approved all animal experiments.

Immunoadsorption

Immunoadsorption was used to eliminate human immunoglobulin from donor blood. Sheep-derived polyvalent anti-human immunoglobulin bound to sepharose was used in a dual-column system according to the manufacturer's instructions (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) with acid-citrate-dextrose (ACD-A) as an anticoagulant diluted 1:20. Separated plasma was passed through the columns.

Perfusion

Porcine and human blood were prepared and perfusion was carried out as previously described [17]. In short, blood for autologous perfusions was freshly taken from the same kidney donor pig and anticoagulated with 1 U/ml heparin (Ratiopharm, Ulm, Germany). For xenogeneic experimental setting, human whole blood (type AB positive) was freshly taken from two healthy donors, heparinized (1 U/ml), and in case of the *xenogeneic* group immediately used for perfusion. In case of the *immunoadsorption* group, blood samples were subjected to the procedure of immunoadsorption prior to perfusion.

Before connecting the kidneys to the perfusion circuit, human C1 Esterase Inhibitor (2 IE/ml; Berinert®; CSL Behring GmbH, Marburg, Germany) was added to the perfusate in all groups to prevent hyperacute rejection. Blood was diluted to a hematocrit of 27% with Tyrodes solution (Sigma-Aldrich, Steinheim, Germany) and Ringer's lactate (Braun-Melsungen AG, Melsungen, Germany) and added to the filtered hard-shell venous reservoir (Affinity Pixie Oxygenation System; Medtronic GmbH, Düsseldorf, Germany) of the perfusion system. Final perfusate and blood volume were adjusted to the weight of perfused porcine kidney. All tubes used in the circuit were coated with heparin (Medtronic GmbH). Perfusion was performed using a roller pump (Stöckert Instrumente GmbH, München, Germany) and a neonatal diffusion membrane oxygenator (Affinity Pixie Oxygenation System; Medtronic GmbH) under physiological conditions with constant pH, pO_2, $pC0_2$, and perfusate temperature. Perfusion parameters [mean arterial pressure (MAP), flow, temperature, blood gases, and hematocrit] were recorded before perfusion and 5, 15, 30, 60, 120, 180, and 240 min after the start of perfusion.

Renal vascular resistance (RVR) was calculated from the following formula:

$$\text{RVR [mmHg/ml/min/g]} = (\text{MAP/flow})/\text{kidney weight.}$$

Endpoint (rejection) of experiment was defined as a 100% increase in RVR compared to the RVR values before perfusion. Graft survival was defined either as time running from start of perfusion until rejection or until termination of the experiment after 240 min.

Blood sampling

At defined time points (0, 5, 15, 30, 60, 120, 180, and 240 min, respectively), blood samples were collected from the venous line of the perfusion system and anticoagulated either with citrate (1/10 vol., 3.2%) or EDTA (1.6 mg/ml; Sarstedt, Nümbrecht, Germany).

Incubation of PAEC with human or porcine plasma

Freshly isolated PAEC (*porcine aortic endothelia cells*) were seeded in 6-well plates at 5×10^5 cells/well and supplemented with Dulbecco's Modified Eagle Mediums (DMEM; Sigma-Aldrich, St. Louis, MO, USA) with the addition of 20% FBS (Biochrom, Berlin, Germany), 1% P/S-Glutamin (Sigma-Aldrich), 25 mM HEPES (Life Technologies, Carlsbad, CA, USA), 50 μg/ml ECGS (BD, Franklin, Lakes, NJ, USA), and 5 g/l D-Glucose (Sigma-Aldrich). Porcine endothelial cells were incubated with human or porcine plasma and supplemented with heparin (1 U/ml). Porcine plasma was used from the same pig for the autologous control and from a different pig for allogeneic control experiment. After an incubation period of 10, 60, and 120 min, supernatants were removed and subjected to measurement of TAT and C3a by an enzyme-linked-immunosorbent assay (ELISA; Siemens HealthCare Diagnostics, Marburg, Germany; Quidel, San Diego, CA, USA).

Coagulation tests

Concentration of D-Dimer was analyzed using a latex particle-based immunoassay (Siemens HealthCare

Diagnostics) and TAT by ELISA (Siemens HealthCare Diagnostics) according to the manufacturer's instructions.

Complement activation, TNF-alpha, IgM, and IgG level

Concentrations of C3a, C5a (Quidel, San Diego, USA), TNF-alpha (R&D Systems, Minneapolis, MN, USA), IgM and IgG (Bethyl-Laboratories, Montgomery, TX, USA) were analyzed by ELISA according to the manufacturer's instructions.

Histology

Kidney biopsies were taken at the end of perfusion. Tissue samples were immediately snap frozen in liquid nitrogen and processed for cryostat sectioning as described before [12]. A standard two-step indirect staining technique using murine anti-human monoclonal antibodies as primary antibodies against human C3a (Dako, Glostrup, Denmark), C5b-9 (Dako, Glostrup, Denmark), IgM (Immunotech Laboratories, Monrovia, CA, USA), IgG, and peroxidase-labeled goat anti-mouse secondary antibodies was performed (Dako, Glostrup, Denmark). 3,3′-Diaminobenzidine (Dako, Glostrup, Denmark) was used as a chromogen before counterstaining with Heamalaun (Merck, Darmstadt, Germany). Deposition was evaluated using a semi-quantitative scoring system (− negative staining, ± weak, + moderate, ++ strong, +++ very strong). In addition, aliquots of obtained biopsies were fixed in formalin (3.7%; Fischar, Merchweiler, Germany) and subjected to standard hematoxylin and eosin staining (Merck, Darmstadt, Germany). For quantification of microthrombi in perfused kidneys, 20 glomeruli per tissue section were investigated and numbers of microthrombi per glomerulum were determined.

Real-time RT-PCR

RNA was isolated from fresh frozen biopsies using NucleoSpin RNA II Kit reagents (Macherey und Nagel, Düren, Germany) according to the manufacturer's instruction. RNA was stored at −80 °C until analysis. Reverse transcription and real-time PCR were carried out as previously described using identical primer sequences [12]. Differential mRNA expression was assessed using the 2-delta CT method [12,25]. Data are presented as x-fold change of expression in relation to baseline (differential mRNA expression from nonperfused kidney from the same donor pig) and normalized to a house-keeping gene (eukaryotic translation elongation factor 1 alpha 1, *EEF1A1*).

Statistical analyses

Results were expressed as the mean ± SEM of at least three independent experiments. Results were analyzed for statistical significance using one-way ANOVA and student's *t*-test using GRAPHPAD PRISM 6.0.

Results

Concentration of human IgM and IgG before and after immunoadsorption

Concentration of human IgM and IgG in the donor blood was assessed in the *xenogeneic* group before perfusion and in the *immunoadsorption* group before and after immunoadsorption prior to perfusion in order to determine the effectiveness of antibody elimination. Level of human IgM antibodies in human donor blood was 589 μg/ml (±160) in the *xenogeneic* group and 946 μg/ml (±244) in the *immunoadsorption* group before immunoadsorption (Fig. 1a). Concentration of human IgG antibodies in human donor blood was 2082 μg/ml (±579) in the *xenogeneic* group and 1703 μg/ml (±218) in the *immunoadsorption* group (Fig. 1a). After immunoadsorption, IgM level decreased in the *immunoadsorption* group to 62 μg/ml (±32). Similarly, IgG level was 166 μg/ml (±107) after immunoadsorption (Fig. 1a). Thus, antibodies were efficiently reduced by immunoadsorption.

Perfusion time and RVR

Perfusion time and renovascular resistance were determined in all groups indicating rejection or obstruction by microthrombosis. Xenogeneic porcine kidney perfusion with human blood resulted in a perfusion time from 120 to 240 min (182 ± 22; Fig. 1b). Hyperacute rejection (within 60 min) was prevented by the use of C1-inhibitor in a high dosage. As demonstrated in Fig. 1b, immunoadsorption of human donor blood (*immunoadsorption*) prior to perfusion did not improve perfusion time (174 ± 28 min). RVR increased during perfusion of porcine kidneys with human blood (*xenogeneic*), but remained constantly low during perfusion of porcine kidneys with porcine blood (*autologous*). Depletion of antibodies resulted in elevated RVR during perfusion time (Fig. 1c). Perfusion time in the *xenogeneic* and the *immunoadsorption* group did not differ significantly, while the course of RVR was different in these both groups.

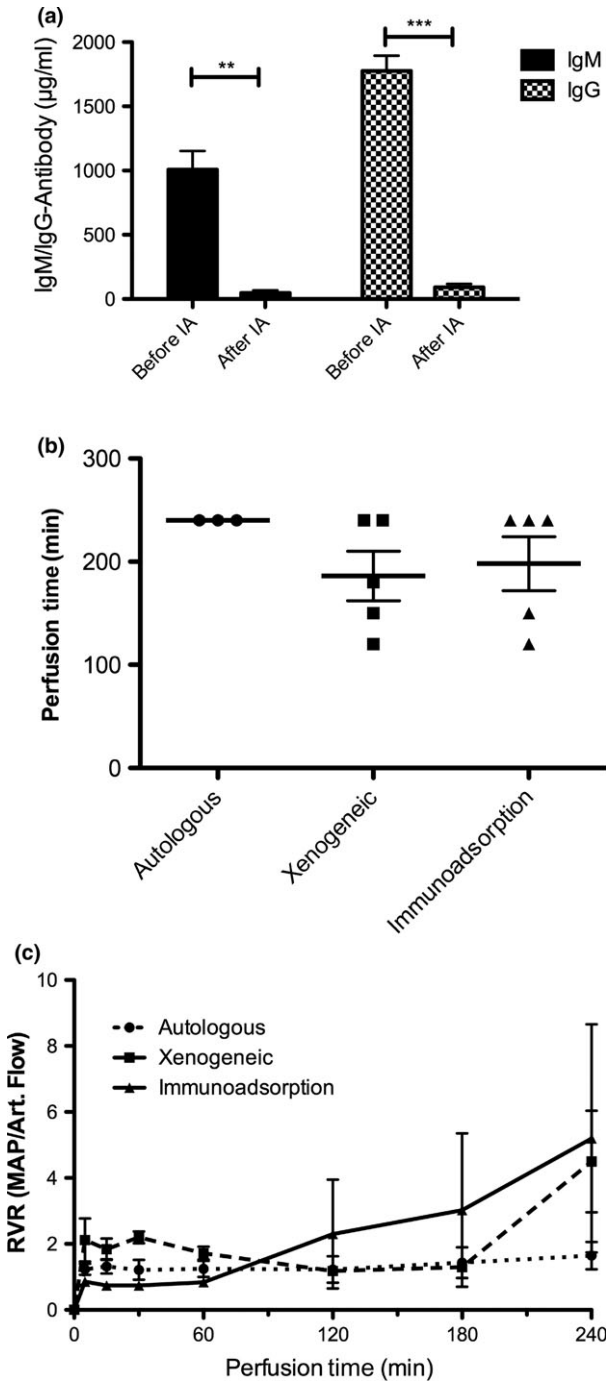

Figure 1 Perfusion time and antibody-level. IgM and IgG levels before and after immunoadsorption (a). Perfusion time of each individual experiment of the three different experimental groups (b). Renovascular resistance (RVR) in the three different experimental groups (c). Results are given as means ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

C3a, C5a, TNF-alpha level

Human C3a, C5a, and TNF-alpha levels were recorded to assess complement activation and inflammation during perfusion. C3a levels increased in the *xenogeneic* group within minutes and remained constantly high during perfusion as shown in Fig. 2a. In contrast, perfusion with immunoadsorbed human blood revealed a constantly low C3a concentration during perfusion (*immunoadsorption*; Fig. 2a). C5a decreased over time after an initial rise in the *xenogeneic* but also in the *immunoadsorption* group (Fig. 2b). As shown in Fig. 2c, concentration of TNF-alpha increased during perfusion in the *xenogeneic* group (213 pg/ml ±79 at 240 min) and remained low in *immunoadsorption* group (38 pg/ml ±5 at 240 min). TNF-alpha and C3a-levels were

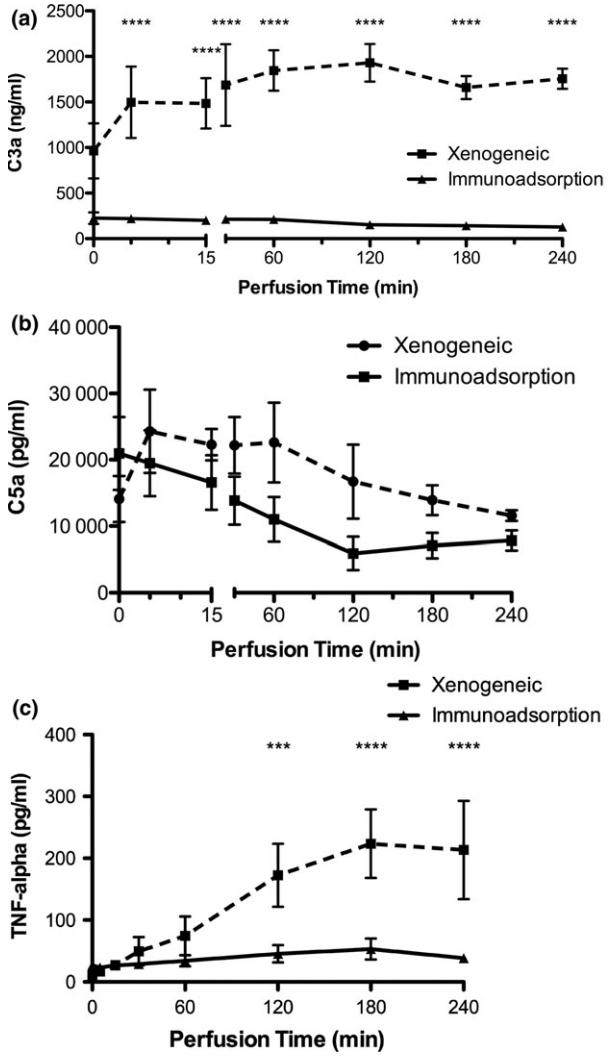

Figure 2 Complement activation and inflammation during xenogeneic perfusion of porcine kidneys. C3a (a) and C5a (b) are shown in the different experimental groups. Human TNF-alpha levels over time in the xenogeneic and the *immunoadsorption* group are shown (c). Results are given as means ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

higher in the *xenogeneic* group compared to the *immunoadsorption* group while C5a levels were similar in both groups at 240 min.

Concentration of D-Dimer and TAT

Activation of coagulation indicated by the concentration of D-Dimer and TAT was recorded over perfusion time in all experimental groups (Fig. 3a and b, respectively). Autologous perfusion revealed a minimal increase of D-Dimer (613 μg/l ±303) and TAT (65 μg/l ±9). In contrast, D-Dimer and TAT raised high during xenogeneic perfusion (22175 μg/l ±7826 and 600 μg/l ±0, respectively). As illustrated in Fig. 3a and b, immunoadsorption could not prevent activation of coagulation as indicated by high levels of D-Dimer (24186 μg/l ±5813) and TAT (566 μg/l ±34) at the end of perfusion.

Activation of coagulation and complement in cell culture assay

To prove that during the perfusion observed xenogeneic activation of coagulation and complement is solely dependent on the interaction between human plasma and the porcine endothelium, porcine aortic endothelial cells (PAEC) were incubated with human plasma without further treatment (*xenogeneic*), with human plasma subjected to immunoadsorption (*immunoadsorption*), with porcine autologous plasma (Fig. 4). Incubation of PAEC with human plasma (*xenogeneic*) showed high elevation of TAT (9999 μg/l ±153; Fig. 4a) and C3a (5593 ± 524 ng/ml; Fig. 4b). In contrast, incubation of PAEC with porcine autologous or allogeneic plasma revealed no activation of coagulation measured by TAT (592 ± 19 and 687 ± 29 μg/l; Fig. 4a). Incubation with immunoadsorbed plasma showed reduced activation of C3a (72 ng/ml ±22; Fig. 4b) but no difference in activation of coagulation as observed by high TAT levels (9533 μg/l ±458; Fig. 4a).

Endothelial cell activation

Activation of porcine endothelial cells indicated by ICAM-1, VCAM-1, and E-Selectin expression after kidney perfusion was assessed by real-time PCR (Fig. 5). Xenogeneic perfusion lead to significant increased expression of VCAM-1 (9.9 ± 2) and E-Selectin (16.5 ± 3.7) compared to *immunoadsorption* and *autologous* group. Autologous perfusion of porcine kidneys with porcine blood resulted in a lower expression of

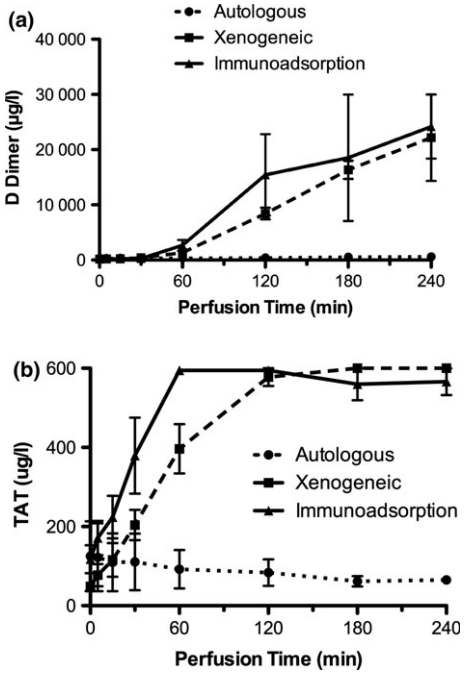

Figure 3 Analyses of aberrant activation of coagulation during *ex vivo* perfusions of porcine kidneys in the three different experimental groups during perfusion. D-Dimer (a) and Thrombin-antithrombin complexes (TAT) (b) were analyzed. Results are given as means ± SEM. P = n.s.

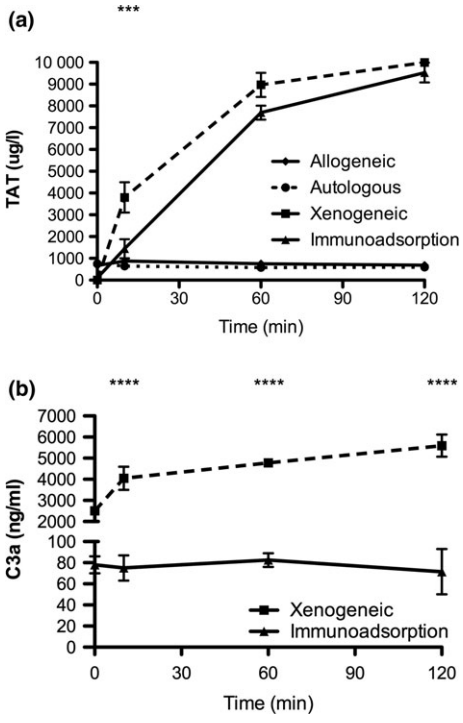

Figure 4 Activation of coagulation by TAT (a) and complement by C3a (b) in cell culture assay. Results are given as means ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

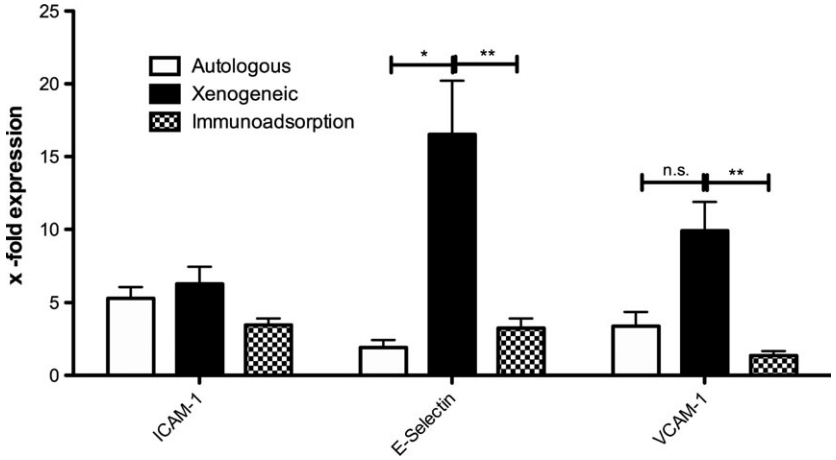

Figure 5 Endothelial cell activation after perfusion in all groups. Endothelial expression of ICAM-1, E-Selectin, and VCAM-1 are shown. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

VCAM-1 (3.4 ± 1) and E-Selectin (1.9 ± 0.5; Fig 5). Endothelial expression of VCAM-1 (1.4 ± 0.3) and E-Selectin (3.3 ± 0.7) was lower in the *immunoadsorption* group compared to the *xenogeneic* perfusions, while the expression of ICAM-1 did not change significantly in the different groups *xenogeneic* (6.3 ± 1.2), *autologous* (5.3 ± 0.8), and *immunoadsorption* (3.5 ± 0.5, Fig. 5).

Deposition of complement and antibodies in perfused kidneys

Deposition of C3a, IgM, and IgG antibodies in perfused kidneys was quantified by immunohistochemistry (Table 1, Fig. 6). Autologous perfusions did not result in deposition of C3a, IgM, or IgG (Fig. 6a–c). Xenogeneic perfusion resulted in deposition of C3a, IgM, and IgG. Deposition of IgM was very strong while C3a was strong and IgG was moderate according to the semi-quantitative scoring (Table 1, Fig. 6d–f). In contrast, in the *immunoadsorption* group, no deposition of antibodies or C3a could be detected (Table 1, Fig. 6g–i).

Occurrence of microthrombi

After perfusion, tissue samples were obtained for detection of microthrombosis. Examination of porcine kidneys perfused with human blood (*xenogeneic*) revealed multiple microthrombi (38 ± 16). Likewise, in the *immunoadsorption* group, multiple microthrombi could be found (46 ± 27). In contrast, few microthrombi were detected after autologous perfusions (2 ± 1). In the *xenogeneic* as well as in the *immunoadsorption* group, multiple microthrombi could be found.

Table 1. Deposition of C3a, IgM, and IgG in the perfused kidneys.

Group	C3a	IgM	IgG
Autologous	–	–	–
Xenogeneic	++	+++	+
Immunoadsorption	–	–	–

Discussion

Xenotransplantation will not be feasible for clinical use until the complex pathology of the occurring rejection processes is fully understood. Although it was longtime thought that in the GalT-KO era antibody-mediated rejection should be no longer a problem, the lesson had to be learned that due to non-Gal antibodies, complement activation and xenogeneic rejection are still a problem [20]. In consequence, soluble (as used in this model) or membrane-bound complement regulation is still needed in *ex vivo* or *in vivo* xenogeneic models [12,15].

Although complement activation can be controlled by transgenic donor pigs expressing human complement regulators (CD59, CD55, and CD46), xenogeneic coagulopathy and the occurrence of intravascular thromboses can be observed and remains a major obstacle to successful xenotransplantation [19]. However, it is still not clear whether aberrant activation of coagulation is a consequence of antibody binding and complement activation or an independent mechanism. There are different studies arguing for both positions [21–24].

In this study, XNA were eliminated and complement activation was inhibited by application of the soluble

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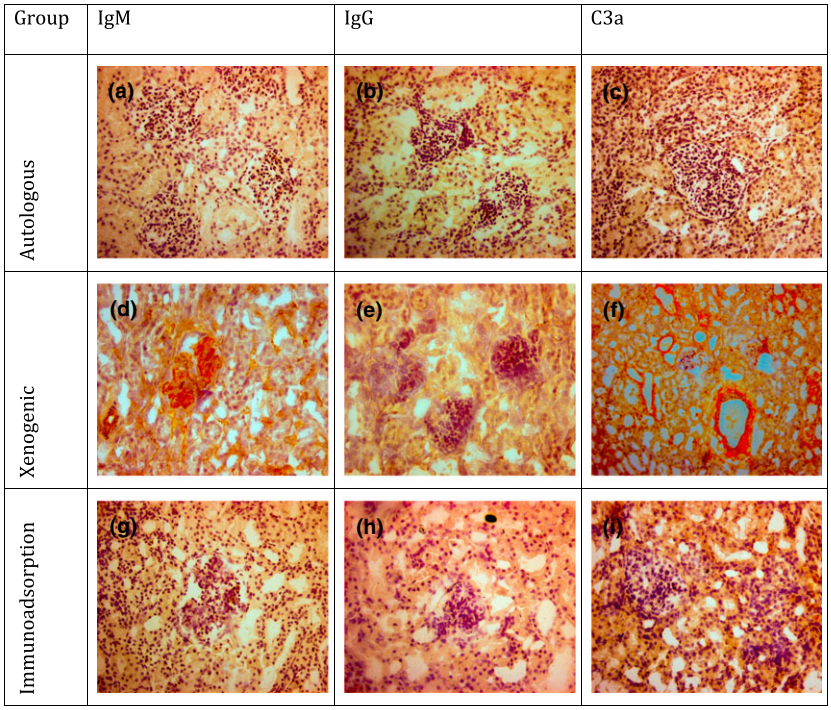

Figure 6 Immunostaining for C3a, IgM, and IgG deposition in porcine kidneys. A representative image of an individual experiment of each group is shown. Porcine kidney after perfusion with porcine blood. No deposits of IgM (a), IgG (b), or C3a (c) can be observed. Porcine kidney after perfusion with human blood. Very Strong IgM (d) and strong C3a (f) as well as moderate IgG (e) deposits can be observed in glomeruli and blood vessels. Porcine kidney after perfusion with human blood in the *immunoadsorption* group. No deposits of IgM (g) IgG (h), and C3a (i) can be observed in glomeruli and blood vessels.

complement inhibitor C1-inhibitor (C1-INH). The use of C1-INH in xenogeneic perfusions was first established by Fiane *et al.* [14]. The *ex vivo* perfusion model used in this study was developed for assessment of coagulopathy based on this concept [12,17].

A limitation of this model that had to be mentioned is that wild-type porcine kidneys were used instead of genetically modified organs expressing human complement regulators in combination with GalKO. The purpose of this study was not to prevent xenogeneic rejection or to evaluate additional treatment strategies, but to investigate the coagulation part of the pathology of AHXR. In this light, we think that the comparability to our previous perfusion studies regarding the coagulation issue is more important than the use of transgenic organs. As our results clearly showed, our model is suitable to address this question. Another important limitation is, that the antibodies were not completely eliminated after immunoadsorption, but reduced to a level that complement activation did not occur. The antibody level after immunoadsorption was low like those in an AB0-incompatible kidney transplantation scenario [25].

C1-INH inhibits complement activation through the classical pathway occurring after binding of antibodies. In contrast, activation mediated by the alternative pathway, as occurring after xenotransplantation of transgenic organs is not regulated [26]. This was confirmed by the elevated C3a-levels during perfusion in the *xenogeneic* group. Therefore, complement activation present in this group contributes to the process of AHXR including coagulopathy. In this model, complement inhibition by the use of C1-inhibitor was adequate, since no HAR occurred.

Our data showed that immunoadsorption of xenoreactive antibodies was sufficient to avoid complement activation as observed by the low C3a levels throughout the perfusions in this group. Although the immunologic part of the AHXR was completely abolished, indicated by low antibodies and C3a levels as well as missing antibody and complement deposition in the perfused kidneys, coagulopathy was not reduced. Coagulation markers D-Dimer and TAT were elevated in the *immunoadsorption* group similar to the *xenogeneic* group. In addition, occurrence of microthrombi was not reduced. These results indicate that xenogeneic coagulopathy has a distinct pathogenesis.

Interestingly, C5a was not reduced by immunoadsorption, suggesting an important link between immunology

and hemostasis [21]. This should be further investigated and be considered as a potential future target for treatment or prevention strategies.

The hypothesis that xenogeneic coagulopathy displays a distinct pathogenesis is supported by data obtained in an *in vivo* study in which the pig hearts transgenic for human CD55 transplanted into baboons were immediately rejected due to dysregulated coagulation [24]. Likewise in our model, organs from Gal-positive pigs were used in this study. The authors have shown that neither complement inhibition via transgenic modification of the donor pigs nor antibody depletion using soluble complexes-forming drugs abolished the activation of coagulation. In line with these findings, our own observations regarding a pig-to-primate kidney xenotransplantation model revealed that despite stable kidney function without any evidence of xenograft rejection, an activation of coagulation could be observed [19]. In another study, even the hearts from GalT-KO pigs transplanted into baboons provoked TMA [9]. Despite a prolonged graft survival, all rejected organs were characterized morphologically by extensive microvascular thrombi, accompanied by deposition of immunoglobulins and complement. These data suggest that the absence of at least anti-Gal antibodies or low anti-Gal antibody titers per se does not prevent the development of microthrombi. Thus, rejection mediated by antibodies and dysregulated coagulation might be two independent forms of response to a xenograft.

Xenogeneic coagulopathy is expected to be a combination of an activated endothelium, platelet aggregation, and molecular incompatibilities of coagulation cascade proteins between the pig and primates or humans [23]. Tissue factor (TF) seems to be crucial for the initiation of coagulation [1,4,7,18]. TF can be expressed on activated endothelial cells as well as on recipient platelets and peripheral blood mononuclear cells (PBMCs) [18,27]. Therewith, TF is not exclusively expressed on endothelial cells activated by XNA and complement, but also on blood cells of the recipient with direct contact to the graft endothelium. Hence, even if the activation of the endothelium is avoided by the reduction of antibody titers, TF expression on recipient′s blood cells could induce activation of coagulation. Monocytes play an important role in this inflammatory-triggered activation of coagulation. After xenogeneic kidney transplantation, a systemic inflammatory response can be observed marked by elevated levels of CRP and IL-6 leading to increased TF expression on monocytes and thrombocytes, resulting in an activation of coagulation [27]. This systemic inflammatory response of the recipient can be downregulated by immunosuppression resulting in delayed activation of coagulation. But even under sufficient immunosuppression, this continuous systemic inflammatory response is still present and remains a hurdle that should be solved [28]. Activation of porcine endothelial cells can be reduced by human thrombomodulin confirming the important role of thrombomodulin by regulating not only coagulation but also endothelial activation in this inflammatory environment [29]. The developing inflammation can be reduced by additional transgenic expression of EPCR, TFPI, CD39, and CD47, modulating coagulation, inflammation, and endothelial cell activation [30]. A long-term kidney survival with 260 days [31] could be achieved by the expression of EPCR/TFPI/CD47 on a GTKO/CD46/CD55 background, addressing the pathology of inflammatory response and TF expression.

Besides inflammation, the molecular incompatibility between pig and human regarding the thrombomodulin/APC-system plays a major role in the development of xenogeneic coagulopathy due to impaired control of coagulation [1,4,7,22]. In recent animal studies, donor pigs expressing human thrombomodulin lead to survival of 136 days for kidney and more than 600 days for heart transplantation without any signs of consumptive coagulopathy, indicating the importance of the regulation of coagulation and especially of the thrombomodulin pathway [16,32].

Recent studies showed that progress had been made in prolonged organ survival by a combination of multitransgenic organs and immunosuppression containing selective receptor-targeting antibodies [33–35]. Particularly, the costimulation blockade by anti-CD40 antibodies plays an important role for long-term cardiac xenograft survival [34]. Combination of anti-CD40 and belatacept could prevent T-cell response [35], indicating the possibilities for future xenotransplantation.

Comparing the results of the heart and kidney pig-to-primate xenotransplantation, the observed coagulopathy appears to be different. Whereas consumptive coagulopathy is more present after xenogeneic kidney transplantation, these extensive systemic coagulopathy is absent after heart transplantation, limiting the coagulopathy to the transplant [36]. From these observations, one would draw the conclusion that the coagulation issue is more important in the kidney and needs to be addressed carefully. Recent data showed that combining the deletion of xenogeneic antigens with the use of recipient animals with low crossmatch reactivity and immunosuppression long-term survival could be achieved [37,38]. In this study, graft survival ranged from 5 to 435 days [37]. The long-term

survivors showed histological signs of glomerulopathy or TMA at the time of explant. These findings are in line with our results that removal of xenogeneic antibodies can prevent activation of complement and innate immunity. However, our results suggest that activation of coagulation cannot be prevented by removing xenogeneic antibodies (or their targets). In this important animal study, coagulation markers were not assessed unlike in our study which would facilitate the analysis of the underlying pathology and therefore remains to be addressed in the future.

Similar to the varying graft survival in this pig-to-primate animal model, we observed in our *ex vivo* perfusion model different responses to the kidney during xenogeneic perfusion, as displayed by varying perfusion times. Although activation of coagulation was always present during xenogeneic perfusion. Therefore, the response of the recipient to donor cells should be determined prior to xenotransplantation. Response intensity of the recipient to the xenograft may determine the degree of inflammation and coagulopathy developing after pig-to-primate or clinical xenotransplantation.

Results of our study suggest that in *ex vivo* perfusions of porcine kidneys, xenogeneic rejection mediated by antibodies and complement activation do not exclusively determine graft survival. Moreover, sufficient control of coagulopathy is needed and warrants prolonged organ survival. From our data, it is hypothetic if C5a is the important link between coagulation and immunology. Supplementary studies are required to elucidate this question. Our data further encourage efforts to focus on the coagulation issue. Thus, our data show that activation of complement C3 is primarily antibody-driven, because it is inhibited by immunoadsorption whereas activation of coagulation remains unaffected thereby indicating a distinct pathogenesis.

Authorship

WR: designed study, collected data, analyzed data, wrote the paper. SW, JK, and MW : analyzed data, wrote the paper. JK, LF, and KJ: performed study, collected data, wrote the paper. SB: performed study, collected data. AT: designed study, analyzed data, wrote the paper.

Funding

DFG TRR 127 “Xenotransplantation”.

Conflict of interest

The authors have declared no conflicts of interest.

REFERENCES

1. Pierson RN 3rd, Dorling A, Ayares D, *et al.* Current status of xenotransplantation and prospects for clinical application. *Xenotransplantation* 2009; **16**: 263.
2. Samstein B, Platt JL. Physiologic and immunologic hurdles to xenotransplantation. *J Am Soc Nephrol* 2001; **12**: 182.
3. Ierino FL, Sandrin MS. Spectrum of the early xenograft response: from hyperacute rejection to delayed xenograft injury. *Crit Rev Immunol* 2007; **27**: 153.
4. Pierson RN 3rd. Antibody-mediated xenograft injury: mechanisms and protective strategies. *Transpl Immunol* 2009; **21**: 65.
5. Breimer ME. Gal/non-Gal antigens in pig tissues and human non-Gal antibodies in the GalT-KO era. *Xenotransplantation* 2011; **18**: 215.
6. Gollackner B, Goh SK, Qawi I, *et al.* Acute vascular rejection of xenografts: roles of natural and elicited xenoreactive antibodies in activation of vascular endothelial cells and induction of procoagulant activity. *Transplantation* 2004; **77**: 1735.
7. Cowan PJ, Roussel JC, d'Apice AJ. The vascular and coagulation issues in xenotransplantation. *Curr Opin Organ Transplant* 2009; **14**: 161.
8. Saethre M, Baumann BC, Fung M, Seebach JD, Mollnes TE. Characterization of natural human anti-non-gal antibodies and their effect on activation of porcine gal-deficient endothelial cells. *Transplantation* 2007; **84**: 244.
9. Shimizu A, Hisashi Y, Kuwaki K, *et al.* Thrombotic microangiopathy associated with humoral rejection of cardiac xenografts from alpha1,3-galactosyltransferase gene-knockout pigs in baboons. *Am J Pathol* 2008; **172**: 1471.
10. Yang YG, Sykes M. Xenotransplantation: current status and a perspective on the future. *Nat Rev Immunol* 2007; **7**: 519.
11. Wu G, Pfeiffer S, Schroder C, *et al.* Coagulation cascade activation triggers early failure of pig hearts expressing human complement regulatory genes. *Xenotransplantation* 2007; **14**: 34.
12. Ramackers W, Friedrich L, Klose J, *et al.* Recombinant human antithrombin prevents xenogeneic activation of hemostasis in a model of pig-to-human kidney transplantation. *Xenotransplantation* 2014; **21**: 367.
13. Alwayn IP, Basker M, Buhler L, Cooper DK. The problem of anti-pig antibodies in pig-to-primate xenografting: current and novel methods of depletion and/or suppression of production of anti-pig antibodies. *Xenotransplantation* 1999; **6**: 157.
14. Fiane AE, Videm V, Johansen HT, *et al.* C1-Inhibitor attenuates hyperacute rejection and inhibits complement, leukocyte and platelet activation in an ex vivo pig-to-human perfusion model. *Immunopharmacology* 1999; **42**: 231.
15. McGregor CG, Ricci D, Miyagi N, *et al.* Human CD 55 expression blocks hyperacute rejection and restricts complement activation in Gal knockout

cardiac grafts. *Transplantation* 2012; **93**: 686.
16. Iwase H, Liu H, Wijkstrom M, *et al.* Pig kidney graft survival in a baboon for 136 days: longest life-supporting organ graft survival to date. *Xenotransplantation* 2015; **22**: 302.
17. Ramackers W, Friedrich L, Tiede A, *et al.* Effects of pharmacological intervention on coagulopathy and organ function in xenoperfused kidneys. *Xenotransplantation* 2008; **15**: 46.
18. Lin CC, Ezzelarab M, Shapiro R, *et al.* Recipient tissue factor expression is associated with consumptive coagulopathy in pig-to-primate kidney xenotransplantation. *Am J Transplant* 2015; **10**: 1556.
19. Hecker JM, Lorenz R, Appiah R, *et al.* C1-inhibitor for prophylaxis of xenograft rejection after pig to cynomolgus monkey kidney transplantation. *Transplantation* 2002; **73**: 688.
20. Ahrens HE, Petersen B, Herrmann D, *et al.* siRNA mediated knockdown of tissue factor expression in pigs for xenotransplantation. *Am J Transplant* 2015; **15**: 1407.
21. Rataj D, Werwitzke S, Haarmeijer B, *et al.* Inhibition of complement component C5 prevent sclotting in an ex vivo model of xenogeneic activation of coagulation. *Xenotransplantation* 2016; **23**: 117.
22. Roussel JC, Moran CJ, Salvaris EJ, *et al.* Pig thrombomodulin binds human thrombin but is a poor cofactor for activation of human protein C and TAFI. *Am J Transplant* 2008; **8**: 1101.
23. Siegel JB, Grey ST, Lesnikoski BA, *et al.* Xenogeneic endothelial cells activate human prothrombin. *Transplantation* 1997; **64**: 888.
24. McCurry KR, Kooyman DL, Alvarado CG, *et al.* Human complement regulatory proteins protect swine-to-primate cardiac xenografts from humoral injury. *Nat Med* 1995; **1**: 423.
25. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; **25**: 402.
26. Miyagawa S, Yamamoto A, Matsunami K, *et al.* Complement Regulation in the GalT KO era. *Xenotransplantation* 2010; **17**: 11.
27. Ezzelarab MB, Ekser B, Azimzadeh A, *et al.* Systemic Inflammation in xenograft recipients precedes activation of coagulation. *Xenotransplantation* 2015; **22**: 32.
28. Iwase H, Liu H, Li T, *et al.* Therapeutic regulation of systematic inflammation in xenograft recipients. *Xenotransplantation* 2017; **24**: e12296.
29. Bongoni AK, Klymiuk N, Wolf E, *et al.* Transgenic expression of human thrombomodulin inhibits HMGB1-induced porcine aortic endothelial cell activation. *Transplantation* 2016; **100**: 1871.
30. Chan JL, Singh AK, Corcoran PC, *et al.* Encouraging experience using multi-transgenic xenografts in a pig-to-baboon cardiac xenotransplantation model. *Xenotransplantation* 2017; **24**: 2.
31. Iwase H, Hara H, Ezzelarab M, *et al.* Immunologic and physiologic observations in baboons with life-supporting genetically-engineered pig kidney grafts. *Xenotransplantation* 2017; **24**: 2.
32. Mohiuddin M, Singh AK, Corcoran PC, *et al.* Genetically engineered pigs and target specific immunomodulation provide significant graft survival and hope for clinical cardiac xeno-transplantation. *J Thorac Cardiovasc Surg* 2014; **148**: 1106.
33. Wang L, Cooper KC, Burdorf L, *et al.* Overcoming coagulation dysregulation in pig solid organ transplantation in nonhuman primates: recent progress. *Transplantation* 2018; **102**: 1050.
34. Mohiuddin M, Singh AK, Corcoran PC, *et al.* Chimeric 2C10R4 anti-CD40 antibody therapy is critical for long-term survival of GTKO.hCD46.hTBM pigt-to-primate cardiac xenograft. *Nat Commun* 2016; **7**: 11138.
35. Iwase H, Ekser B, Satyananda V, *et al.* Pig-to-baboon heterotopic heart transplantation exploratory preliminary experience with pigs transgenic for human thrombomodulin and comparison of three costimulation blockade-based regimens. *Xenotransplantation* 2015; **22**: 211.
36. Cowan PJ, Robson SC. Progress towards overcoming coagulopathy and hemostatic dysfunction associated with xenotransplantation. *Int J Surg* 2015; **23**: 296.
37. Adams AB, Kim SC, Martens GR, *et al.* Xenoantigen deletion and chemical immunosuppression can prolong renal xenograft survival. *Ann Surg* 2018; **268**: 564.
38. Meier RPH, Muller YD, Balaphas A, *et al.* Xenotransplantation: back to future. *Transpl Int* 2018; **31**: 465.