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Inhibition of platelet aggregation by the GPIIb/IIIa antagonist Reopro does not significantly prolong xenograft survival in an ex vivo model

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Abstract Effects on hyperacute rejection were studied in a discordant model with the platelet GPIIb/IIIa antagonist Reopro. Pig kidneys perfused with human blood survived median 118 min in the Reopro group and 103 min in the controls ($P = 0.22$). Platelet and leukocyte counts decreased, whereas plasma thrombospondin and soluble as well as platelet membrane P-selectin increased significantly in both groups without significant intergroup differences. β-Thromboglobulin and myeloperoxidase increased significantly more in the control group than in the Reopro group ($P = 0.009$ and $P = 0.02$, respectively). The classical complement pathway was substantially and similarly activated in both groups. Light and electron microscopy revealed arterial thrombi and numerous glomerular platelet aggregates in the control group in contrast to the Reopro group. In conclusion, Reopro reduced platelet aggregation, and platelet and leukocyte activation to some extent, but had no effect on complement activation and did not significantly prolong xenograft survival, even though better preservation of morphology was shown.

Key words Xenotransplantation · Hyperacute rejection · Thromboregulation · GPIIb/IIIa antagonist

Introduction

In hyperacute rejection (HAR), xenograft loss is associated with xenoreactive antibody deposition, complement activation, and activation of platelets and coagulation with microvascular thrombosis [9, 17, 28–30]. Endothelial cells are the first to be damaged [4], and platelets are activated by exposure to subendothelial collagen, von Willebrand factor, thrombin, or activated complement [3]. P-selectin (CD62P) expression and secretion of ADP and serotonin by activated platelets may promote leukocyte infiltration and localized vaso-

constriction and thereby contribute to malfunction of the xenograft [7]. The complement cascade can initiate clotting [5, 35], and platelets may be activated by complement factor C1q with an induction of GPIIb/IIIa and the expression of surface-bound CD62P with a procoagulant activity [25]. At least in humans, the membrane form of the terminal complement complex (TCC, SC5b-9) may induce vesiculation of the endothelial plasma membrane and thereby expose a catalytic surface for the prothrombinase enzyme complex, which can contribute to fibrin deposition associated with immune endothelial injury [15].

The effects of platelet inhibition with dipyridamole, aspirin, ticlopidine, prostanoids, dextran, and heparin have been relatively limited in xenograft models [33]. The major platelet integrin GPIIb/IIIa is a transmembrane glycoprotein fundamental to the formation of platelet aggregates and potentiating adhesion to subendothelial matrices via fibrin(ogen), von Willebrand factor, and vitronectin. Interference by selective GPIIb/IIIa receptor antagonism should expectedly counteract thrombosis. In vitro results have suggested that there may be "pathological" or nonphysiological activation due to crucial incompatibilities between pigs and humans in the regulation of human platelet reactivity and hemostasis [18, 21, 24]. Anti-GPIIb/IIIa agents do not necessarily have the same efficacy across designated species barriers, and various GPIIb/IIIa antagonists may show clear differences [8]. Pareti and colleagues [24] showed that pig von Willebrand factor-induced platelet aggregation in humans was only partly blocked by an anti-GPIIb/IIIa monoclonal antibody (LJCP8), which totally inhibits the binding of fibrinogen and other adhesive molecules to stimulated human platelets. On the other hand, Candinas et al. [7] have given evidence that interference with platelet aggregation by using GPI 562, a selective and specific GPIIb/IIIa antagonist, prolongs xenograft survival when guinea pig hearts are heterotopically transplanted into Lewis rats. In contrast, Robson et al. [29] found no benefits with respect to cardiac function and transplant survival when using GPI 562 in an ex vivo discordant pig heart model.

The objective of this study was to determine the effect of using the specific GPIIb/IIIa antagonist Reopro on discordant pig-to-human xenograft kidney survival and assess the ability of this agent to modulate activation of platelets, leukocytes, and complement in an ex vivo perfusion setup. Our results suggest that Reopro to some extent depressed platelet and leukocyte activation but had no significant effect on the activation of complement or on graft survival.

Materials and methods

Experimental groups, perfusion device, and procedure

Six Norwegian landrace pigs of either sex, with a median body weight of 21 kg (range 19–23 kg), were obtained from a local farmer and supplied with food and water ad libitum. The study was carried out at a Laboratory Animals Unit approved by the National Animal Research Authority for such work on pigs. The protocol was approved by the Unit's Competent Person, according to the regulations (1996) established by the Norwegian Animal Welfare Act of 1974. Kidneys were removed using sterile procedures as previously described [11]. Immediately after isolation, the organs were perfused with precooled Ringer's acetate and kept on crushed ice. Prior to ex vivo perfusion, cannulae were inserted into the renal artery and the renal vein. The good biocompatibility of the ex vivo perfusion system, resulting in physiological conditions of the pig kidneys, has previously been described in detail [11]. In the test group, pig kidneys ($n = 6$) were perfused with human blood to which Reopro was added, and, in the control group, pig kidneys ($n = 6$) were perfused with human blood supplied with human serum albumin (HSA). The total fluid volume, protein concentration, and amount of blood were equal in both groups. The perfusion was performed in both circuits simultaneously with pooled blood from the same two donors.

Reagents

Reopro (Abciximab; Centocor, Leiden, The Netherlands) is the Fab fragment of the chimeric (humanized) mouse monoclonal antibody 7E3. Reopro inhibits aggregation of platelets by inhibiting the binding of fibrinogen, von Willebrand factor, vitronectin, and other adhesive molecules to the GPIIb/IIIa receptor on activated platelets [26]. Although inhibition of platelet aggregation is likely to be the major effect of Reopro, it probably also decreases formation of thrombin [26]. We used Reopro at a final concentration of 0.7 mg/ml (corresponding to 0.58 mg/kg human body weight), given as a bolus dose, based on the following in vitro tests: A blood platelet aggregometer (Aggro-meter; Chrono-Log, Havertown, Pa., USA) equipped with fluorescence facilities was used to measure the inhibitory effect of Reopro on platelet aggregation in human blood. Platelet-rich plasma (PRP) was prepared from citrated whole blood (9 vol of whole blood, 1 vol of 0.11 M trisodium citrate) by centrifugation at 300 *g* at room temperature for 10 min. Reopro was added to one aliquot at a concentration of 0.7 mg/ml. Platelet-poor plasma (PPP) was made from PRP by centrifugation at 2000 *g* for 20 min. Aggregation studies were performed at 37 °C, stirring at 1200 r.p.m., setting light transmission through PPP to 100 % and light transmission through PRP to 0 %. Fifty microliters of Chrono-lume reagent, which is a luciferin/luciferase reagent sensitive to ATP (Chrono-Log), was added to two aliquots of 400 µl of PRP with or without Reopro. Subsequently 50 µl of either collagen (8.4 µg/ml, final concentration), ADP (9.37 µM, final concentration), or thrombin receptor-activating peptide (TRAP, 25 µM, final concentration) was added, recording change in light transmission and increase in fluorescence per minute. In control samples, collagen and TRAP induced full aggregation and ATP release, while addition of ADP gave first wave aggregation, but no ATP release. In samples spiked with Reopro, none of the above agonists caused aggregation, but full ATP release was noted in the TRAP-challenged sample. It can thus be concluded that Reopro completely blocked platelet aggregation in the concentrations used. HSA (Octapharma, Hurdal, Norway) was added in the same protein concentration and volume as Reopro in the control perfusion setup.

Table 1 Platelet variables before, at the beginning (*T0*) and after 15 and 60 min of perfusion and at rejection in kidneys perfused with Reopro or HSA

	Reopro group ($n = 6$)		HSA group ($n = 6$)		Reopro vs. control group
	Median (95% CI)	P-value[a] vs. before	Median (95% CI)	P-value[a] vs. before	P-value[b]
Platelets ($\times 10^9$/l)					0.33
Before	146 (93–200)	–	147 (95–200)	–	
T0	116 (79–164)	NS	101 (58–166)	NS	
T15	81 (51–126)	< 0.001	56 (31–79)	< 0.05	
T60	78 (59–96)	< 0.001	49 (29–74)	< 0.001	
Rejection	82 (55–109)	< 0.001	43 (22–66)	< 0.001	
BTG (ng/ml)					0.009
Before	864 (574–1277)	–	939 (602–1323)	–	
T0	723 (544–838)	NS	1261 (874–1631)	NS	
T15	970 (683–1332)	NS	1802 (1077–2536)	< 0.05	
T60	1253 (557–1989)	NS	1965 (670–3320)	NS	
Rejection	1168 (698–1904)	NS	2991 (2344–3680)	< 0.001	
Soluble CD62P (ng/ml)					0.07
Before	55 (39–78)	–	55 (39–78)	–	
T0	64 (42–98)	NS	69 (36–124)	NS	
T15	80 (63–92)	< 0.01	80 (53–175)	< 0.05	
T60	87 (65–129)	0.001	100 (78–127)	< 0.001	
Rejection	103 (77–130)	0.001	176 (126–308)	< 0.001	
Thrombospondin (ng/ml)					0.18
Before	166 (60–417)	–	166 (60–417)	–	
T0	177 (95–425)	NS	246 (136–467)	NS	
T15	157 (113–480)	NS	272 (208–542)	< 0.01	
T60	238 (189–510)	NS	406 (323–500)	< 0.0005	
Rejection	369 (262–772)	< 0.001	957 (510–1498)	< 0.0005	

[a] Friedman test, [b] ANOVA; BTG, β-thromboglobulin; HSA, human serum albumin

Blood samples and analyses

Blood samples were collected from the perfusion circuits before and immediately after the addition of Reopro or HSA, at 5 min, 15 min, and 60 min after the beginning of perfusion, and at rejection. Serum and plasma were stored at ÷ 70 °C until analysis.

β-Thromboglobulin (BTG) was determined as earlier described [38]. Thrombospondin was quantified using a double-antibody enzyme immunosorbent assay (EIA). Microtiter plates were coated overnight with the monoclonal anti-thrombospondin antibody (clone P12; Immunotech, Marseilles, France) at 0.4 µg/ml. The standard for this assay was a pool of calibrated serum from 20 healthy blood donors. Plasma samples were obtained as described for BTG and used at a 1:2 dilution. A biotinylated monoclonal anti-thrombospondin antibody (clone P10; Immunotech) was used for detection, and final development was performed using streptavidin-conjugated horseradish peroxidase (Amersham, Buckinghamshire, UK) and the substrate 2,2 × -azino-di-(3-ethyl)-benzthiazoline sulfonic acid (ABTS; Boehringer Mannheim, Mannheim, Germany).

Samples for quantification of soluble CD62P (sP-selectin) were obtained as described for BTG and assayed with a CD62P EIA kit (Bender MedSystems, Vienna, Austria). Samples for myeloperoxidase (MPO) were drawn in tubes containing ethylenediaminetetraacetic acid (EDTA). MPO was assayed in EIA as described [37].

Complement activation products were quantified in EDTA plasma by EIAs based on neoepitope-specific monoclonal antibodies. Activation of the classical complement pathway was measured by assays detecting complexes between C1rs and C1-inhibitor (C1rs-C1inh) [13] and C4 activation products (C4bc) [39], whereas activation of the final common pathway was measured using assays for C3 activation products (C3bc) and terminal SC5b-9 complement complex (TCC) [23].

Correction for hemodilution

The blood cell counts and the concentration of BTG, soluble CD62P, thrombospondin, MPO, and complement activation products were corrected for hemodilution by using hematocrits [6].

Flow cytometry

Flow cytometry was performed as described elsewhere [12]. Platelets were identified by their distinct patterns on forward scatter and 90° side scatter and by their expression of CD41 (GPIIb/IIIa receptor). Platelet CD62 and CD41 levels were quantified by a double-staining technique using a PE-conjugated monoclonal anti-CD41 and a fluorescein isothiocyanate (FITC)-conjugated anti-CD62 (Central Lab Netherlands Red Cross, Amsterdam, The Netherlands).

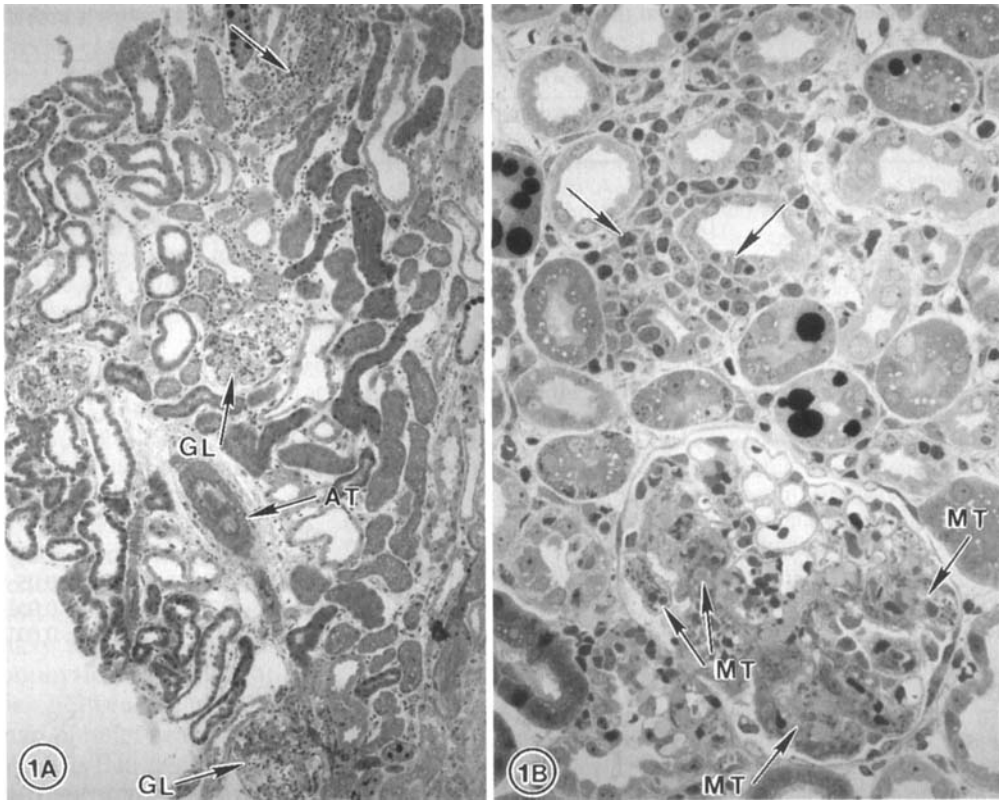

Fig. 1 a,b Light micrographs of biopsies from control organ. **a** Artery with a thrombus (*AT*) and glomeruli (*GL*) with microthrombi. Leukocyte infiltrate (*arrow*; × 100). **b** Detail of glomerulus with microthrombi (*MT*). Leukocyte infiltrate with tubulitis (*arrows*; × 400)

Light microscopy, conventional electron microscopy, and immune electron microscopy

Kidney biopsies were obtained ex vivo by excision at rejection. For light microscopy and conventional electron microscopy, about 0.5-mm^3 cubes of cortical tissue were fixed by immersion for 4 h in 2% glutaraldehyde in 0.1 M phosphate buffer and postfixed in 1% osmium tetroxide in the same buffer for 2 h. The specimens were further processed as described elsewhere [2, 11, 12]. For immune electron microscopy, the specimens were fixed in 2% paraformaldehyde and 0.05% glutaraldehyde in the same buffer, with further processing as described in [12]. The immunolabeling was carried out according to the method described by Robertson et al. [27]. The primary antibody was polyclonal antibody to human TCC, a rabbit antiserum produced in our laboratory. The primary antibody was detected by anti-rabbit IgG conjugated to 5-nm gold particles (Amersham) diluted 1:50. Silver enhancement was obtained using a silver enhancing kit (Amersham) for 6 min. After washing, the sections were contrasted by standard procedures with uranyl acetate and lead citrate. Control studies were carried out omitting the primary antibody. The specimens were analyzed in a Jeol 1200 EX electron microscope.

Statistics

Results are given as medians and 95% confidence intervals. Data were first analyzed by two-way repeated-measures analysis of variance (ANOVA) using the SPSS-PC program package. Due to differences in the duration of individual experiments and the occurrence of nonnormal variables and unequal variances, the conditions for two-way repeated-measures analysis of variance were only partly met. Therefore, the Friedman test was used for subsequent comparisons of parameter changes over time within each group, and the Mann-Whitney *U*-test was employed for subsequent comparisons between groups. In order to achieve an overall *P*-value for significance below 0.05 and correct for multiple comparisons, any *P*-values from the Friedman or Mann-Whitney *U*-tests below the corresponding *P*-values from the repeated-measures two-way ANOVA were regarded invalid.

Results

Graft survival and gross morphology

The survival time in the Reopro group was 118 min (90–150 min) and in the control group 103 min (88–120 min; $P = 0.22$). The pig kidneys had normal color at the start of the experiments, then gradually became darkly mottled in appearance. Ureter peristalsis continued until rejection in all experiments.

Platelets

The platelet counts (Table 1) decreased significantly with time ($P < 0.001$) in both groups, without significant intergroup differences ($P = 0.33$). BTG did not increase

Table 2 Leukocyte variables before, at the beginning (*T0*) and after 15 and 60 min of perfusion and at rejection in kidneys perfused with Reopro or HSA

	Reopro group ($n = 6$)		HSA group ($n = 6$)		Reopro vs. control group
	Median (95% CI)	P-value[a] vs. before	Median (95% CI)	P-value[a] vs. before	P-value[b]
Leukocytes ($\times 10^9/l$)					0.15
Before	3.5 (2.8–4.3)	–	3.5 (2.8–4.3)	–	
T0	3.3 (2.2–4.7)	NS	2.9 (2.1–3.6)	< 0.001	
T15	3.0 (1.9–4.2)	< 0.05	2.3 (1.5–3.1)	< 0.001	
T60	1.3 (1.0–2.3)	< 0.001	1.5 (1.0–2.1)	< 0.001	
Rejection	1.5 (1.2–2.0)	< 0.001	0.9 (0.7–1.1)	< 0.001	
Neutrophils ($\times 10^9/l$)					0.34
Before	1.7 (1.2–2.1)	–	1.7 (1.2–2.1)	–	
T0	1.7 (0.8–2.3)	NS	1.5 (0.7–2.3)	NS	
T15	1.3 (0.6–2.1)	NS	0.9 (0.4–1.5)	< 0.0005	
T60	0.7 (0.2–1.3)	< 0.0005	0.4 (0.1–0.7)	< 0.0005	
Rejection	0.8 (0.3–1.1)	< 0.0005	0.2 (0.1–0.5)	< 0.0005	
Myeloperoxidase ($\mu g/l$)					0.02
Before	325 (152–705)	–	678 (137–1223)	–	
T0	354 (181–1152)	NS	1221 (673–1936)	< 0.05	
T15	1084 (368–1732)	< 0.05	2420 (1722–2743)	< 0.0005	
T60	1831 (1258–2530)	< 0.001	3233 (2580–3773)	< 0.0005	
Rejection	2455 (1269–4007)	< 0.001	4783 (3252–9813)	< 0.0005	

[a] Friedman test, [b] ANOVA; HSA, human serum albumin

significantly with time in the Reopro group, in contrast to the HSA group ($P < 0.001$), and the intergroup difference was significant ($P = 0.009$). Soluble CD62P increased significantly ($P < 0.001$) in both groups during perfusion, with a trend of less increase in the Reopro group ($P = 0.07$). Thrombospondin increased significantly in both groups ($P < 0.001$); however, there was not a significant difference between the groups ($P = 0.18$). There was a small increase in CD62P and CD41 expression on platelets in the HSA group ($P < 0.0005$ and $P < 0.01$, respectively), as opposed to the Reopro group, but the intergroup differences were not significant, probably due to large variations in the data ($P > 0.45$; data not shown).

Leukocytes

The total leukocyte, neutrophil, lymphocyte (not shown), and monocyte (not shown) counts (Table 2) decreased significantly with time in both groups, without significant intergroup differences. MPO concentrations increased significantly in both groups ($P < 0.001$), and the increase was significantly lower in the Reopro group ($P = 0.02$).

Complement activation products

The concentration of C1rs-C1inh complexes, C4bc, C3bc, and TCC increased significantly and equivalently in both groups from the beginning of perfusion to rejection, without significant differences between the groups.

Microscopy

Light microscopy of sections from Epon-embedded kidney biopsies revealed marked differences between the HSA and the Reopro groups. In the HSA group, numerous platelet thrombi were observed in glomerular capillaries (Fig. 1 a,b), often together with mono- or polymorphonuclear leukocytes. A few arteries also contained thrombi (Fig. 1 a). Foci of leukocyte infiltrates with tubulitis were seen (Fig. 1 a,b). The tubular epithelium showed various changes, often with vacuolization, but the changes varied from region to region. In the Reopro group, the glomerular capillaries showed no or only a few loosely packed aggregates (Fig. 2 a,b), and arterial thrombi were not observed. The other changes were similar but less pronounced than those in the control group.

By electron microscopy, the capillary platelet aggregates observed in the HSA group showed various degree of packing but were often closely packed with varying amounts of fibrin (Fig. 3 a), in contrast with the Reopro group (Fig. 3 b). Capillary endothelial damage was

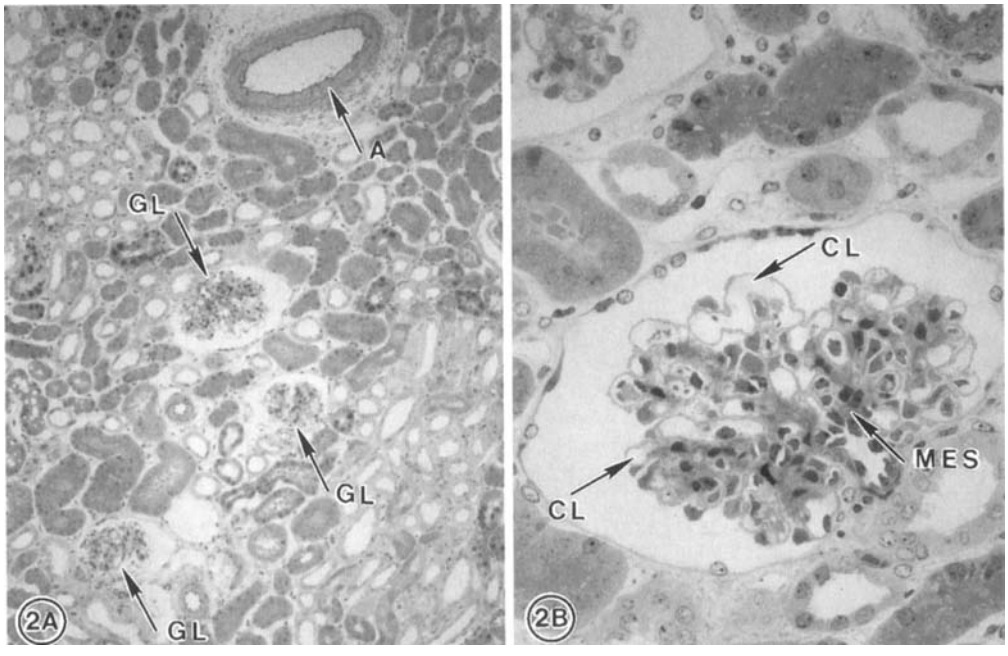

Fig. 2 a, b Light micrographs of biopsies from organ perfused with Reopro. **a** Glomeruli (*GL*) and artery (*A*) with no or few microthrombi (× 100). **b** Detail from a glomerulus with open capillary lumina (*CL*). Mesangium (*MES*; × 400)

observed with cytoplasmic swelling (Fig. 4 a). Platelets often adhered to the capillary wall. As shown in Fig. 4 b, arterial thrombi with fibrin were found. The tubular epithelial changes varied from region to region but were characterized by swollen mitochondria, vacuolization, simplification of the basal foldings, and loss of cytoplasmic matrix. The Reopro group showed glomerular capillaries, sometimes containing leukocytes, but platelet aggregates were few, and no fibrin was observed in association with the platelets. The platelets were "activated" with numerous pseudopods and disturbance of the internal organelles, and adhesion to the endothelium was seen. The tubular changes were similar to those in the control group but appeared less pronounced. Immunoelectron microscopy revealed that TCC was located in the glomerular capillary lumina, in the basement membrane and mesangial matrix (Fig. 5 a,b), and even in the urinary space. The amount of labeling varied somewhat from glomerulus to glomerulus and also from experiment to experiment, but there was no difference between the HSA and Reopro group. Labeling was also seen in phagosomes of the tubular epithelium in both groups.

Discussion

To our knowledge, this is the first report in which Reopro has been studied in a discordant ex vivo perfusion model of pig kidneys.

Perfusion setup

The ex vivo perfusion system we used has been extensively discussed in an earlier publication [11]. We used Ringer's acetate, which is a mild vasodilator, and heparin in our setup, to prevent clotting in the extracorporeal circuit. Both modulations may have prolonged perfusion before rejection. Heparin may influence both complement, platelet, and leukocyte activation [31, 32]. However, the heparin concentration and Ringer's acetate were similar in both groups and therefore cannot explain the observed differences between the groups. In clinical studies, concurrent administration of high doses of heparin may give hemorrhage of Reopro [1], but this was not seen in our study.

Dose of Reopro used in the study

Whether the Reopro concentration of 0.7 mg/ml was optimal for effective inhibition of platelets in our ex vivo setup may be a subject for discussion. In the preliminary tests (see Materials and methods), the drug concentration was effective in blocking human platelet aggregation in vitro. Microscopy also revealed marked differences in platelet aggregation in the Reopro and control groups, suggesting that Reopro was active, although a complete block was not obtained. The drug concentration in our setup was chosen on the basis of experiences in human studies [36] and should sufficiently block all GPIIb/IIIa receptors with minimal loss of Reopro during the experiment. Candinas et al. [7] found increased

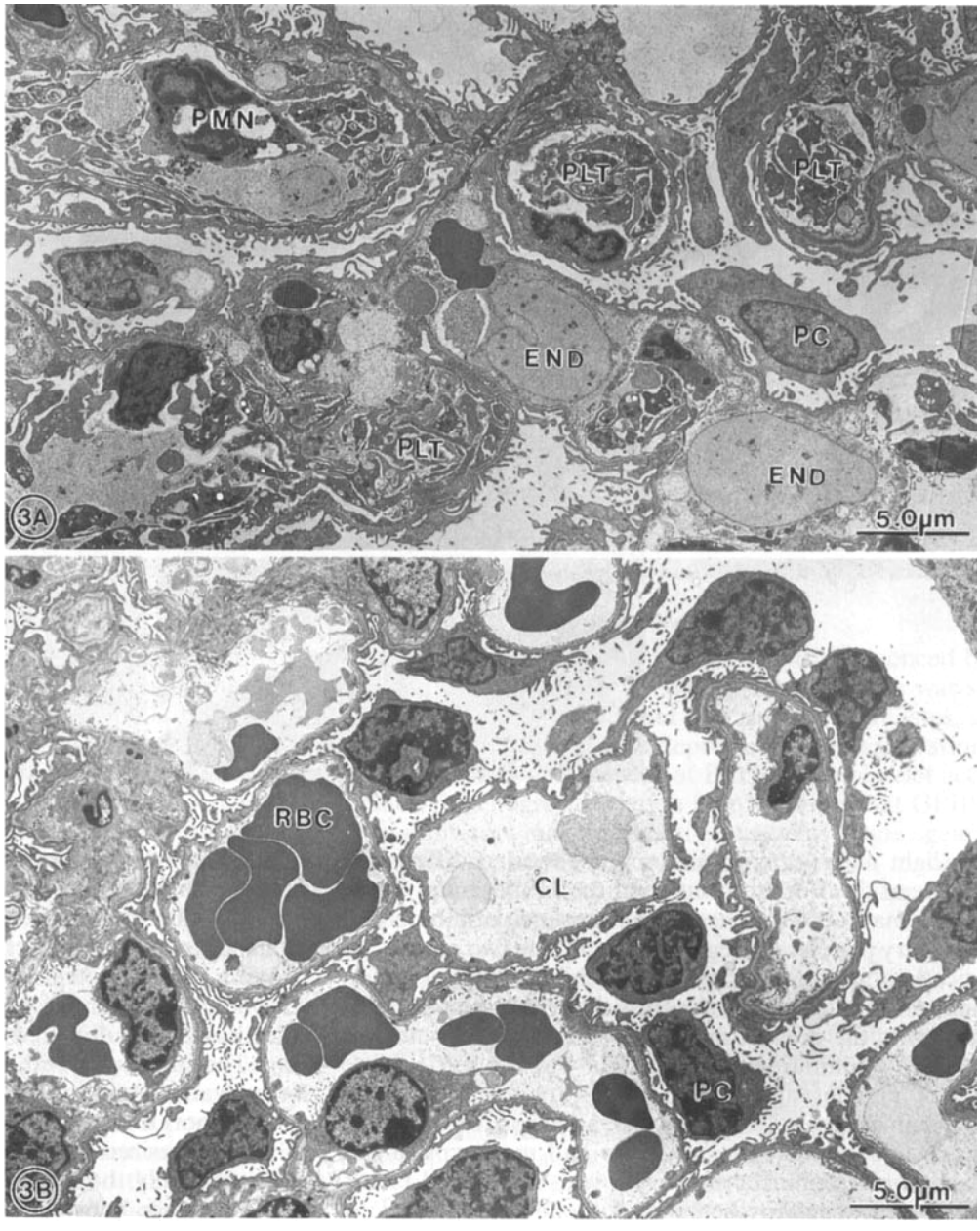

Fig. 3 a, b Electron micrographs. **a** From control organ with glomerular capillaries filled with platelet aggregates (*PLT*) and some leukocytes (*PMN*; *END* swollen endothelial cells, *PC* podocyte). **b** From Reopro perfused organ with glomerular capillaries containing some red cells (*RBC*), but no platelet aggregates. Capillary lumen (*CL*)

graft survival at a high dose (0.5 mg/kg) in their discordant small animal xenograft model using GPI 562, another selective GPIIb/IIIa antagonist that completely inhibited platelet aggregation in vitro. A low dose (0.1 mg/kg) partially inhibited platelet aggregation without prolonging graft survival. Thus discrepancies between species in efficiency of GPIIb/IIIa antagonists might be of importance. In contrast to Candinas et al. [7], Robson et al. [29] found that platelet counts remained stable throughout the procedure without increase in graft survival in their discordant pig-to-human ex vivo perfusion model also using GPI 562 (0.5 µM).

Effects of Reopro on platelet activation markers and formation of thrombi

At the microscopic level, there are differences in the constituents of the thrombus both between allogeneic and xenogeneic HAR and between the xenograft reaction in different species combinations [19]. Extrapolation of mechanisms from one species combination to another must therefore be done with caution.

Microthrombi were observed in small vessels in both groups at rejection by electron microscopy, consistent with thrombosis as an important issue of HAR. Howev-

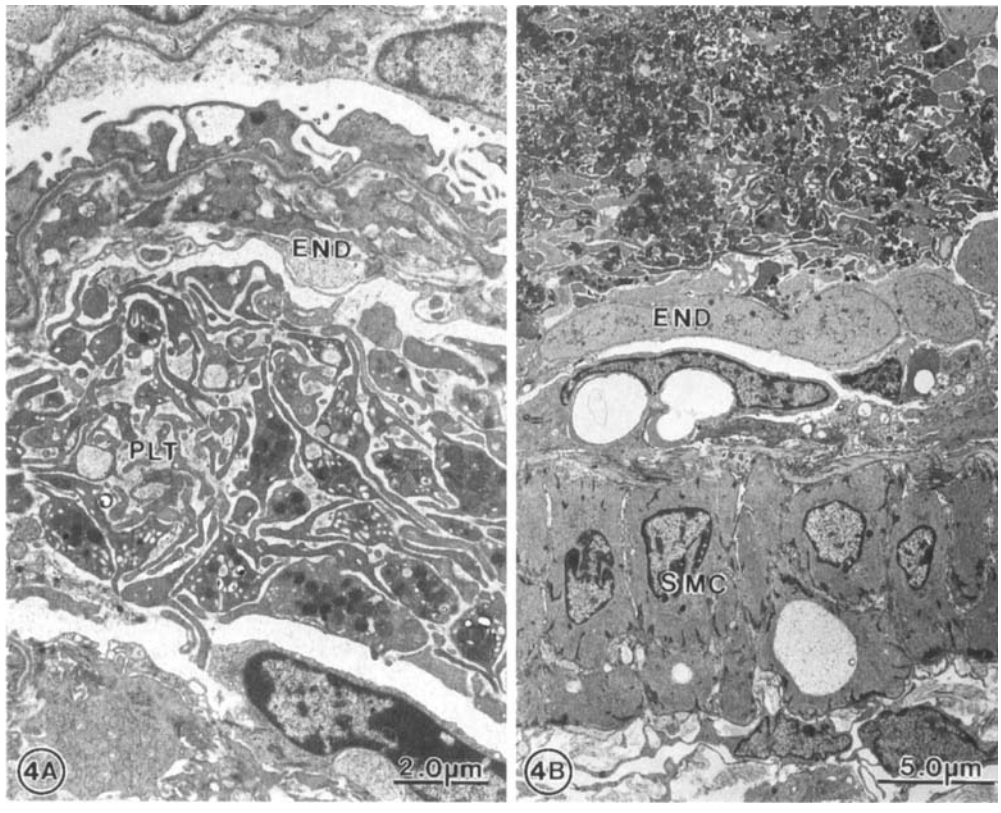

Fig. 4 a Detail of platelet aggregate from control organ with numerous platelet pseudopods (*PLT*) and altered endothelium (*END*). **b** Artery with swollen endothelium (*END*) and thrombus material with platelets and fibrin. Smooth muscle cells (*SMS*)

er, light and electron microscopy revealed differences between the Reopro group and the HSA group. There was a marked difference in the presence of glomerular capillary platelet aggregates between the two groups, indicating that the Reopro had an inhibitory effect on formation of such aggregates. It is likely that platelet activation in the present model is so vigorous that close to complete inhibition of GPIIb/IIIa was not sufficient to block platelet aggregation. Other platelet activation mechanisms, e.g., through the adhesion receptor Ib, were not inhibited in our setup and may explain this lack of effect. Furthermore, pig von Willebrand factor, produced by microvascular endothelium, is structurally different from human von Willebrand factor and can bind directly to and activate GPIb and GPIIb/IIIa receptors [14]. Likewise, thrombin-initiated assembly of TCC potentiates thrombin-stimulated platelet aggregation, and assembly of TCC on the platelet plasma membrane results in increased prothrombinase activity without lysis. Furthermore, xenoantibody and complement cause rapid cleavage of heparin sulfate proteoglycan from the endothelial cell. These factors could contribute to amplification of HAR [17].

There was a marked reduction in platelet counts during perfusion in both the test and the Reopro group. The reduction was less pronounced in the Reopro group, but the difference between the groups was not statistically significant. An explanation for this may be that the adhesion of platelets to the endothelial and artificial surface is similar in both groups, since Reopro does not inhibit adhesion but aggregation of platelets. The total number of platelets contained in the aggregates as shown by electron microscopy may be limited compared with those being depleted from the circulation by adhesion. The substantial reduction of the fluid-phase platelet activation-marker BTG with Reopro is consistent with a report from a human study [22]. It should, however, be emphasized that Reopro inhibits aggregation but not specifically the release reaction from the platelets. The difference between BTG and thrombospondin release may be that thrombospondin is not platelet specific, in contrast to BTG, since thrombospondin also can be produced by activated endothelial cells [20].

Effects of Reopro on leukocytes, complement, and graft survival

Significantly less MPO was liberated in the Reopro group than the control group. We suggest that Reopro had a modulatory effect on neutrophils, probably secondary to the effect on platelets, leading to less adhesion, extravasation, and release of MPO. Neutrophils accumulate in platelet-rich thrombi at sites of vascular

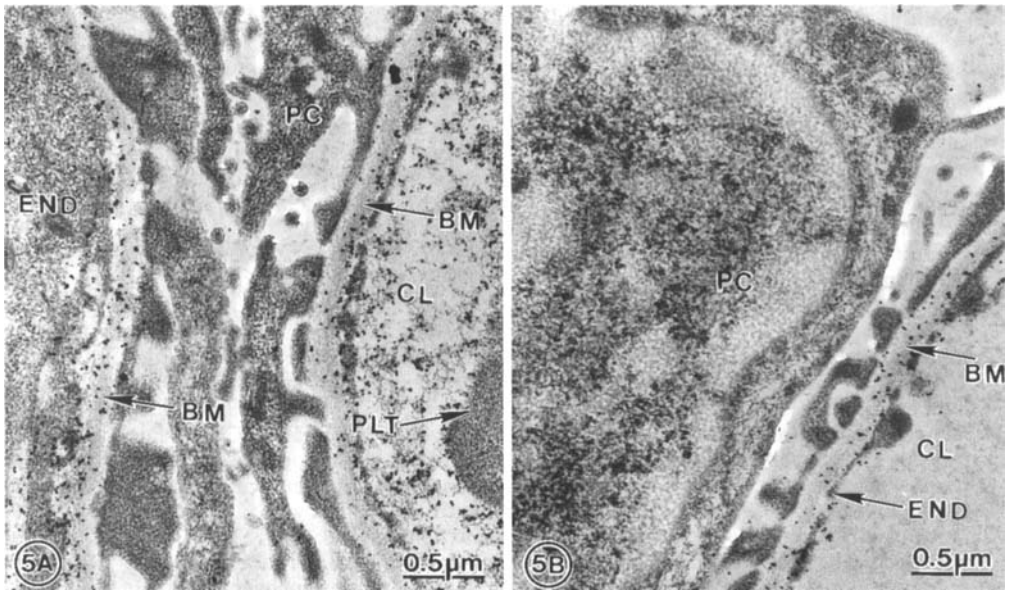

Fig. 5 a Electron micrograph from silver-enhanced, gold-labeled anti-TCC, showing labeling of basement membrane (*BM*) and the capillary lumen (*CL*). Edge of platelet (*PLT*). Endothelium (*END*). **b** From Reopro-perfused material with staining of TCC on basement membrane (*BM*) and endothelium (*END*; *PC* podocyte)

injury. However, inhibition of platelet aggregation only delays but does not prevent leukocyte recruitment [16]. The adhesion of platelets to neutrophils may modify the activity of both cell types during hemostasis and inflammation [10]. Furthermore, it is well known that Reopro directed against the platelet glycoprotein IIb/IIIa cross-reacts with the leukocyte integrin CD11b and thus directly blocks neutrophil activation and adhesion to fibrinogen and ICAM-1 [10, 34].

The similar increases in C1rs-C1inh, C4bc, C3bc, and TCC in both groups without significant intergroup differences demonstrate that Reopro did not influence complement activation, which is a main component of HAR in this xenograft model. The fluid-phase similarities between the two groups with respect to complement were paralleled in the electron-microscopic observations, showing equal amounts of TCC deposition. Our ultrastructural studies indicated complement fixation, platelet aggregation, and adhesion to the damaged endothelial surfaces, with formation of microthrombi, and the migration of erythrocytes and neutrophils into the interstitium.

Survival was not significantly prolonged in the Reopro group. This finding does not disregard platelets as mediators of HAR, and it cannot be excluded that a beneficial effect could be obtained in combination with other therapeutic strategies.

Conclusion

The process of xenograft rejection is complex. The present study showed, for the first time in a pig-to-human ex vivo perfusion model of xenotransplantation, that pig kidney survival was not significantly influenced by adding the GPIIb/IIIa antagonist Reopro. This was so even if Reopro led to fewer and smaller aggregates, as shown by microscopy, and depressed platelet activation, as indicated by less release of BTG. Thus, platelet activation in xenograft rejection is so vigorous that GPIIb/IIIa inhibitors may be of limited value as single agents. To achieve prolonged survival of xenografts, more effective inhibition of inflammatory pathways, especially the complement system, seems necessary.

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