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Inhibition of platelet activation in human clinical renal ischemia/reperfusion

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Abstract

Apart from their primary function in haemostasis, platelets serve as modulators of inflammation. Although in preclinical studies activated platelets have been implicated in the inflammatory response after reperfusion, their role in clinical renal ischemia/reperfusion (I/R) injury is unknown. In this study, the role of platelets in living, brain dead and cardiac dead donor kidney transplantation was assessed by our unique method of arteriovenous measurements over the reperfused kidney. Markers of platelet activation and degranulation, i.e. β-thromboglobulin, soluble glycoprotein Ib and platelet derived growth factor did not show an arteriovenous concentration difference over the reperfused kidney. The solitary RANTES release from brain dead donor kidneys presumably reflects leukocytic release. Since no overt platelet activation was observed, more subtle changes in excitability of platelets were measured. Remarkably, platelets in renal venous blood were less easily and less intensely excitable than platelets in arterial blood. In conclusion, results of this study unequivocally deny platelet activation in early reperfusion in both living and deceased donor kidney transplantation. Platelet excitability was even inhibited in the reperfused kidney, suggesting platelets do not initiate the inflammatory response of renal I/R injury.

Introduction

Ischemia/reperfusion (I/R) is an important clinical problem and an inevitable component of organ transplantation. I/R injury is characterized by inflammation,[1-6] but the mechanism that initiates this inflammatory reaction is unknown. At present no effective therapy is available. A role of platelets in the etiology of I/R injury has been suggested but is not fully sustained or understood.[7] Upon activation, platelets release granules containing growth factors, cytokines, chemokines and leukotrienes into the circulation by exocytosis.[8] As such, platelets serve as inflammatory mediators that can initiate recruitment and activation of leukocytes and thereby significantly aggravate tissue injury.[9]

Increasing experimental evidence suggests that platelet activation orchestrates an inflammatory response upon reperfusion.[10, 11] However, clinical studies on the mechanism and timing of platelet activation in I/R injury are scarce. In myocardial infarction, platelets mediate thrombotic occlusion and increase damage by causing micro vascular occlusions and no-reflow phenomenon.[12] Administration of anti-platelet agents is routine therapy in acute coronary syndrome.[13] Whether platelet activation, apart from its role in infarction and thrombosis, is involved in the initiation of inflammation in clinical I/R injury in humans is not clear yet.

In this study, kidney transplantation was used as model of human I/R injury. Since peripheral blood measurements are influenced by many systemical influences, and factors of interest are diluted in the total intravascular volume, we instead applied selective arteriovenous measurements over the reperfused graft to specifically assess local release of mediators. The role of platelet activation and excitability was systematically investigated using these arteriovenous measurements over the reperfused kidney. We show that there is no platelet activation in both living, brain dead and cardiac dead donor kidney transplantation in the immediate period following reperfusion. In fact, platelet excitability is even repressed in the reperfused kidney, indicating that regulatory mechanisms prevent post-reperfusion platelet activation and subsequent thrombus formation. It is therefore unlikely that platelets are an initiator of the inflammatory response leading to I/R injury.

Patients and Methods

Patient population

Thirty-four patients undergoing renal allograft transplantation were included for arteriovenous sampling. Of these, 8 patients received a kidney from a living donor, 9

patients received a kidney from a brain dead donor, and 7 patients received a kidney from a cardiac dead donor. Brain dead and cardiac dead donors together are referred to as deceased donors. Ten other patients that received a kidney from a living donor were included for direct arteriovenous measurements of platelet excitability, and biopsy collection for immunohistochemical anlysis. Patient characteristics are shown in Table 1. For technical reasons (renal vein sampling), only patients receiving a left kidney were included. The study protocol was approved by the local ethics committee, and informed consent was obtained from each patient.

Table 1: Transplantation and outcome characteristics in living donor (LD), brain dead donor (BDD) and cardiac dead donor (CDD) kidney transplantation.

	LD PEA, IHC	LD	BDD	CDD
n	10	8	9	7
Donor age: mean (SD)	53.1(10.0)	43.9(10.6)	54.1(17.1)	52.7(15.3)
Donor gender (M:F %)	30:70%	75:25%	44:56%	43:57%
Preservation fluid	HTK (n=10)	HTK (n=8)	UW (n=9)	UW(n=2) HTK (n=5)
WIT1 in min. (SD)	N/A	N/A	N/A	23.1(7.7)
CIT in h. (SD)	2.8(0.4) * ∇	3.0(0.3) * ∇	19.7(6.2)	17.3(2.6)
WIT2 in min. (SD)	30.9(8.1)	34.0(6.3)	33.0(6.1)	34.1(6.4)
Recipient age: mean (SD)	47.8(16.1)	41.1(10.5)	55.1(13.5)	54.0(11.2)
Recipient gender (M:F %)	70:30%	38:62%	44:56%	71:29%
Creatinine clearance day 30 (ml/min)	49.8(6.1) ∇	73.3 (20.5) * ∇	49.3(15.3) ∇	27.1(10.3)
DGF (%)	0%	0%	56%	86%
DGF: dialysis after transplantation in days (SD)	0 (0) * ∇	0 (0) * ∇	7.0 (5.3) ∇	17.2 (7.2)

LD procedures that were exclusively sampled for platelet excitability assay (PEA) and biopsy collection (IHC) are presented in a separate column. WIT1: first warm ischemia time, CIT: cold ischemia time, WIT2: second warm ischemia time, DGF: delayed graft function. * P <.05 compared to BDD, ∇ P <.05 compared to CDD.

Operation and materials

Kidney transplantations were performed according to local standardized protocol. In living donors minimally invasive nephrectomy was performed. For cold perfusion and storage of the kidney, Custodiol® Histidine-tryptophan-ketoglutarate solution (HTK) solution (Tramedico, Weesp, The Netherlands) was used. Deceased donor kidneys were perfused and stored with University of Wisconsin solution (UW) or HTK (Table 1). The immunosuppressive regimen was based on induction therapy with an interleukin-2 receptor blocker and maintenance treatment with tacrolimus or cyclosporine A, in addition to mycophenolate mofetil and steroids in all groups. During surgery, no heparin or other drugs influencing platelet

aggregation were administered. Donors, not recipients, were routinely administered a daily prophylactic dose of low molecular weight heparin.

Arterial and renal venous blood samples were obtained as described before in detail.[14] In short, via a small catheter placed in the renal vein, 10 ml blood aliquots were sampled at 30 seconds, 1, 3, 5, 10, 20 and 30 minutes after reperfusion (i.e., t=0). Paired arterial blood samples were obtained at 0, 3, 5, 10, 20 and 30 minutes after reperfusion (Figure 1). All samples were collected in tubes containing EDTA and immediately placed on ice. Blood samples were centrifuged (1,550 *g*, 20 min, 4ºC) and the derived plasma was subsequently centrifuged to deplete it from leukocytes and platelets (1,550 *g*, 20 min, 4ºC). Plasma was aliquotted and stored at -70°C until assayed. A renal cortical biopsy was obtained after cold storage, and a second biopsy of the same kidney was collected 45 minutes after reperfusion.

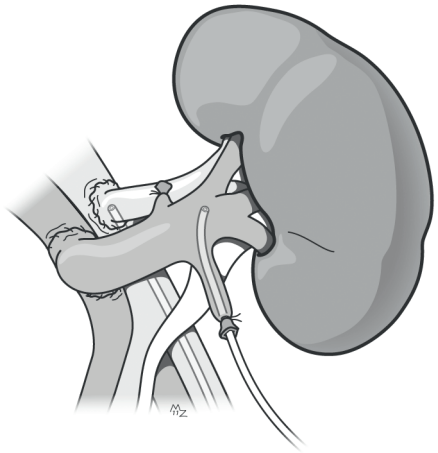

Figure 1. Schematic representation of the arteriovenous sampling method over the reperfused kidney by simultaneous blood collection from the renal artery and vein. Illustration by Manon Zuurmond© (www.manonproject.com).

Plasma measurements

Platelet activation was assessed by measuring the local renal release of platelet granule contents into the circulation. Platelets contain three main types of secretory granules: the α-granules (the most prominent population in size and number), the dense granules and the lysosomal granules. Beta-thromboglobulin (β-TG) is abundant in α-granules and is a specific marker for platelet activation. Levels of β-TG and soluble GPIb (sGPIb) were determined in plasma using semi-automated ELISA on a TECAN Freedom EVO 150. Each antigen was measured on a separate Nunc maxisorb 384 well ELISA plate (Thermofischer Scientific, Roskilde, Denmark). The capture antibodies, MAB393 (1 µg/mL) and mouse anti-GP1b (clone 6.30; 0.9 µg/mL) were coated on two different plates for 2 hours. Unbound antibodies were

removed by washing five times using phosphate-buffered saline (PBS)/0.5% Tween. Plasma samples were diluted 1/80 for NAP-2 and 1/25 for sGPIb measurements and added to the plate with the corresponding capture antibody. Each plate contained four calibration curves consisting of standard serum sample with known β-TG and sGPIb concentrations. Dilutions were made in PBS/1% BSA and incubated for 2 hours on the capture antibodies. Unbound antigens were removed by washing five times with PBS/0.5% Tween. The detection antibodies BAF393 (50 ng/mL) and biotinylated M1852 (0.25 µg/mL, Sanquin Amsterdam) were added to the corresponding plates. Unbound detection antibody was removed by three wash steps with PBS/0.5% Tween, after which streptavidin/horseradish peroxidase (HRP) was added for 2 hours to bind the biotin on the detection antibody. Bound antigen was quantified using Supersignal West Pico Chemiluminescent Substrate (#34080, Thermoscientific, Rockford USA) on a Spectramax Luminescence Microplate Reader (Molecular Devices, Sunnyvale, USA). The chemokine 'Regulated on Activation, Normal T Cell Expressed and Secreted' (RANTES) and platelet-derived growth factor (PDGF)-BB can be released from platelet α-granules upon activation. Both RANTES and PDGF-BB were measured in a custom made multiplex assay in accordance with the manufacturer's instructions (X-plex, Biorad, Veenendaal, the Netherlands).

Platelet excitability assay (PEA)

In the absence of platelet activation, more subtle changes in excitability of platelets were explored, using a renewed, bedside applicable assay for platelet excitability. For this purpose, arterial and renal venous samples from another 10 patients undergoing living donor kidney transplantation were collected in citrate coated tubes at 5 and 30 minutes after reperfusion. Whole blood was added to tubes containing either platelet agonist adenosine diphosphate (ADP), collagen-related peptide (C-RP) or thrombin related activatable peptide (TRAP) in eight increasing concentrations. Fluorescent anti-P-selectin and anti-GP1b antibody were co-incubated for subsequent flow cytometry analysis. Final concentrations in the tubes were 0.01, 0.03, 0.12, 0.49, 1.95, 7.81, 31.25, 125.00 µM ADP; 0.2, 0.6, 2.4, 9.8, 39.1, 156.3, 625 and 2500 ng/ml C-RP; and 0.04, 0.15, 0.61, 2.44, 9.77, 39.06, 156.25, 625.00 µM TRAP. After 20 minutes of incubation, the reaction was stopped by adding 0.5% formaldehyde in heparin buffered saline. FACS-Calibur flow cytometer adjusted in a standard configuration (Cytomics FC 500 flow cytometer, Beckman and Coulter, Krefeld, Germany) was used to assess the activation state and excitability of platelets. Samples were analyzed on GP1b positivity (all platelets) and P-selectin positivity (activated platelets). A typical example of the flow cytometry result of a low and high percentage of activated platelets is shown in Figure 2A and 2B. Analysis involved assessment of the maximum percentage of activated platelets (% P-selectin positive) and their intensity of activation (mean fluorescence index; MFI). The percentage activated platelets increased with concentration of the agonist in a sigmoid shaped curve (Figure 2C).

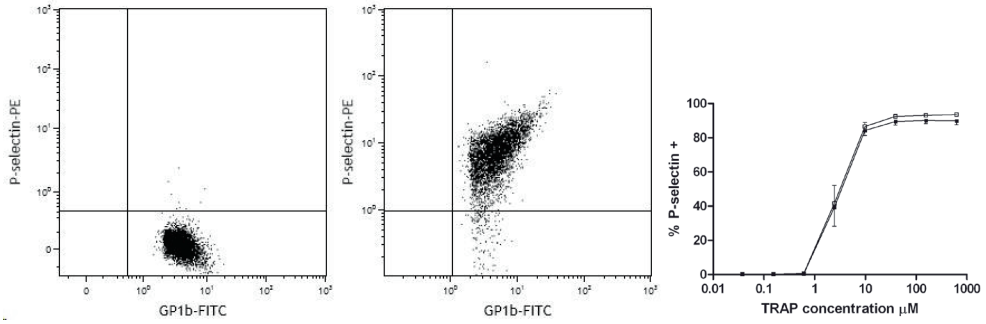

Figure 2. Typical examples of flow cytometry results after stimulation with low (A) and high (B) concentration agonist, i.e. thrombin related activatable peptide (TRAP). The corresponding P-selectin positive percentage of platelets was 0.1% and 96.6%, respectively. The eight results of a stimulation series are depicted in sigmoid shaped curves were arterial □ and renal venous ■ percentages of P-selectin positive platelets can be compared (C).

Platelet staining

Pre-transplant and reperfusion (45 min. post reperfusion) cortical biopsies were collected from living donor kidneys. Biopsies were snap frozen and cryostat sections (5 µm) were fixed in acetone for 10 min. Endogenous peroxidase activity was blocked with 0.1% H_2O_2. Slides were incubated with primary antibody biotin labeled CD41 antibody (ab30434, Abcam, Cambridge, United Kingdom), followed by ABC complex (Vectastain, Vector laborotories, Peterborough, United Kingdom). The staining was visualized using DAB (DAKO, Glostrup, Denmark). Sections were counterstained with hematoxylin (Merck, Darmstadt, Germany).

Analysis

Clinical donor data were retrieved from Eurotransplant Foundation (Leiden, The Netherlands). Delayed graft function (DGF) was defined as the need for dialysis within one week after transplantation. Statistical analysis was performed using SPSS 16.0 statistical analysis software (SPSS Inc, Chicago, Ill). The area under the curve (AUC) was calculated for the arterial and venous curve of the plasma measurements for the total of 30 minutes. The delta AUC was calculated (venous minus arterial) and the null-hypothesis (delta AUC is 0) was tested by t-test or non-parametric test in case of a non-normal distribution. Wilcoxon test was applied to test for differences between arterial and venous outcome per agonist and per time-point in the analysis of the PEA. Graph error bars represent the standard error of the mean (SEM). A *P*-value of less than 0.05 was considered significant.

Results

Donor and transplant characteristics

Recipient and donor age and gender were similar in all three donor groups (Table 1). As expected, the warm and cold ischemia duration differed between the groups, with a shorter duration of cold ischemia in living donor kidney transplantation. The immunosuppressive regimen was similar in the three groups. A significantly higher rate of DGF was observed in brain dead and cardiac dead donor groups as compared to living donor kidney transplantation. Although incidence of DGF was similar in brain dead donor and cardiac dead donor kidney transplantations, DGF was more prolonged in the cardiac dead donor group. With the exception of one kidney from a cardiac dead donor in which the recipient was not compliant with immunosuppressive medication, all kidneys were still functioning at one year after transplantation. There were no complications because of local or deep venous thrombosis or any (re-)bleeding problems.

No platelet activation in the reperfused kidney

Platelet activation leading to degranulation was assessed by comparing plasma concentrations of granule contents, i.e. RANTES, PDGF-BB, ß-TG and GP1b between arterial and renal venous samples, i.e. net change over the kidney. RANTES was not released from the kidneys of living or cardiac dead donors (P=0.11, P=0.34 respectively). Kidneys of brain dead donors however, showed a significant RANTES release during the first 30 minutes of reperfusion (P=0.03, Figure 3). PDGF-BB was not released from living, brain dead, or cardiac dead donor kidneys (P=0.28, P=0.51, P=0.14, respectively). Similarly, ß-TG and GP1b were not released from the kidney in any of the three donor groups (P=0.68, 0.72, 0.17 for ß-TG, and P=0.08, 0.64, 0.51 for GP1b in LD, BDD and CDD respectively).

Inhibition of platelet excitability in the reperfused kidney

Although direct arteriovenous measurements over the kidney did not indicate platelet activation and degranulation in the reperfused kidney, more subtle changes in platelet excitability may be induced when platelets pass through the graft. Maximum platelet excitability was measured by an established platelet activation assay, now optimized for bedside use. Before stimulating the samples, there was no arteriovenous difference in percentage of spontaneously P-selectin positive platelets at 5 minutes (arterial 0.24%, venous 0.22%; P=0.36) or 30 minutes after reperfusion (arterial 0.25%, venous 0.23%; P=0.21). After in vitro stimulation of arterial or renal venous blood with platelet activation agonists ADP, C-RP and TRAP, the maximum total percentage of activated platelets and the mean fluorescence index (MFI) of activated platelets were measured. There was a significant decrease in maximum percentage activated platelets over the kidney for both

C-RP (P=0.01) and TRAP (P=0.005) at 5 minutes after reperfusion. At 30 minutes after reperfusion this venous decrease in activation was not significant anymore (Figure 4). The MFI of activated platelets was significantly lower in renal venous blood compared to arterial blood at 5 minutes after reperfusion for both C-RP (P=0.047) and TRAP (P=0.009). After 30 minutes, only ADP activated platelets showed a significant decrease in MFI (P=0.013, Figure 5). Overall results of the platelet excitability assay indicate that platelets are less easily and less intensely excitable after they passed the reperfused kidney.

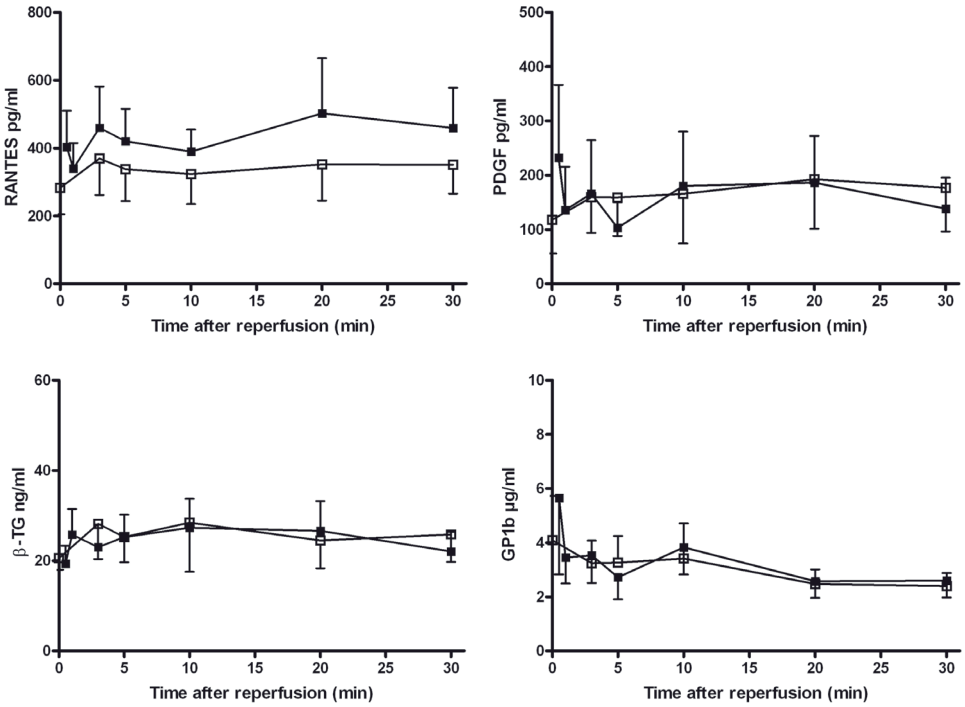

Figure 3. Arterial □ and venous ■ concentrations of (A) RANTES, (B) PDGF, (C) ß-TG and (D) GP1b are shown for the first 30 minutes of reperfusion of brain dead donor kidney grafts. RANTES was significantly released (P=0.03), however other platelet degranulation markers were not released from the kidney. Graph error bars represent the standard error of the mean (SEM).

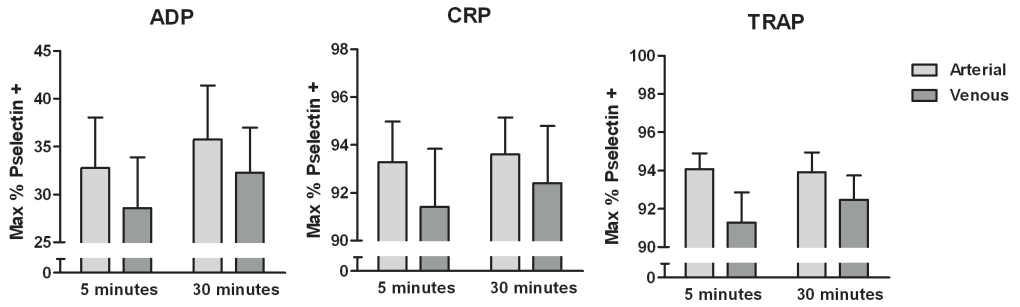

Figure 4. Maximum percentage activated platelets after stimulation with agonists adenosine diphosphate (ADP), collagen-related peptide (C-RP) or thrombin related activatable peptide (TRAP). Five minutes after reperfusion there was a significant decrease in maximum percentage activated platelets over the kidney for both C-RP (P=0.013) and TRAP (P=0.005). After 30 minutes of reperfusion changes were not statistically different anymore. Graph error bars represent the standard error of the mean (SEM).

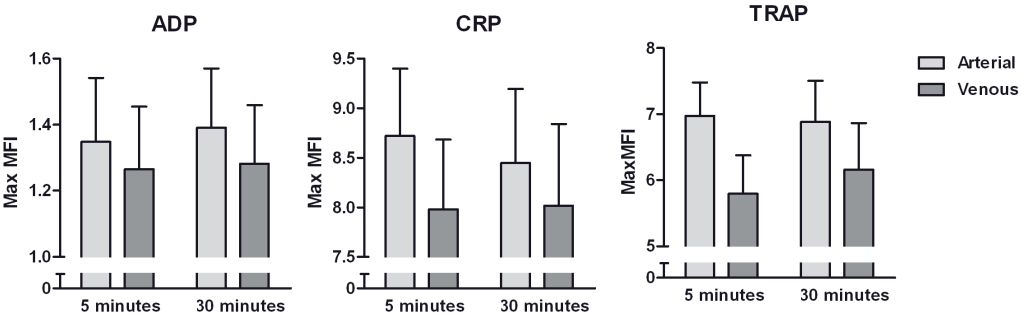

Figure 5. Maximum intensity of activated platelets after stimulation with agonists adenosine diphosphate (ADP), collagen-related peptide (C-RP) or thrombin related activatable peptide (TRAP). Five minutes after reperfusion there was a significant decrease in maximum intensity over the kidney for both C-RP (P=0.047) and TRAP (P=0.009). After 30 minutes of reperfusion only ADP activated platelets showed a significant decrease in intensity (P=0.013). Graph error bars represent the standard error of the mean (SEM).

No platelet retention in the kidney

The difference between arterial and venous platelet excitability could be explained by retention of a subpopulation of (activated) platelets in the kidney. To evaluate platelet retention, arterial and renal venous platelet concentrations were measured 5 and 30 minutes after reperfusion. There was no difference between arterial and renal venous concentration of platelets at 5 minutes after reperfusion (mean arterial 148.6 x 10^9/l, renal venous 145.1 x 10^9/l, P=0.08) and at 30 minutes after reperfusion (mean arterial 147.6 x 10^9/l, renal venous 145.2 x 10^9/l, P=0.29), and therefore retention of platelets in the kidney is unlikely. To

substantiate the lack of platelet infiltration in the reperfused kidney, immunohistochemical analysis of renal biopsies collected before and 45 minutes after reperfusion was executed as well. CD41 staining showed occasional clusters of platelets, mostly in glomeruli (Figure 6). Pre- and post-reperfusion patterns were highly similar, and thereby substantial platelet retention after reperfusion could be excluded.

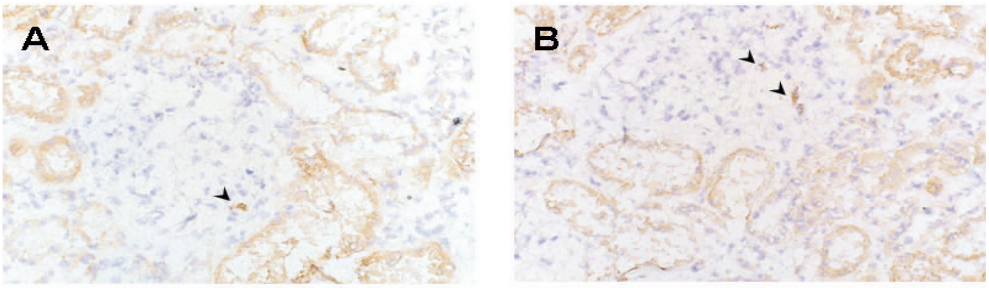

Figure 6. Representative sections showing CD41 staining in living donor renal biopsy tissue collected (A) pre, and (B) post-reperfusion. Occasional CD41 positive cells were detected, mostly in glomeruli (arrows). Original magnification 200x.

Discussion

Platelets have been suggested to be involved in experimental I/R injury. However, their potential role in human I/R injury and the opportunities of anti-platelet agents in clinical I/R injury are unclear. In this study, we show there is no platelet activation in early I/R injury in human kidney transplantation. Moreover, platelet activation is even repressed in renal venous blood, suggesting a platelet inhibitory effect of the reperfused kidney.

It is well-established that platelets, aside from their thrombotic role, are contributors to the inflammatory response through release of cytokines, chemokines and growth factors from their granules.[15] Platelets have been suggested to be involved in the inflammatory response of I/R injury in various organs. In mouse gut, platelets roll and adhere to post-reperfusion endothelium in a P-selectin dependent mechanism.[16] Furthermore, I/R injury of the liver results in platelet adhesion to the sinusoidal endothelium.[17-21] The first activated platelets are present in mouse myocardial tissue within two minutes after reperfusion,[22] and then accumulate in the infarcted myocardium.[23] In the mouse renal vascular bed, platelet accumulation has been shown early after reperfusion as well.[24] However, the role of platelet activation in the initiation of human clinical I/R injury in general is unclear. In kidney biopsies potential infiltration of P-selectin positive platelets after reperfusion was not consistent.[25] The many intervention studies using anti-platelet agents in myocardial

infarction show beneficial effects, however it is difficult to discern the effects of diminished (microvascular) thrombosis from potential anti-inflammatory effects.

In this study, platelet activation upon reperfusion was examined in living and deceased donor kidney transplantation. Since deceased donor kidneys have more clinical organ damage, platelet activation was expected to be more pronounced in these kidneys. In the arteriovenous measurements, renal release of various platelet degranulation products into the circulation was assessed. Among all of the measured factors, only RANTES was found to be minimally released from the reperfused kidney of brain dead donors. Since none of the other platelet degranulation markers were released from the reperfused kidney, it is more likely that RANTES is released from another source. Increased T-lymphocyte and macrophage content is found in kidneys from brain dead donor grafts already before transplantation, and both cell types can be a source of RANTES.[1] Moreover, RANTES is constitutively released during the first 30 minutes of reperfusion, while a peak after reperfusion could be expected should the source of this RANTES be degranulating platelets. Since none of the other platelet activation markers were released in any of the groups, platelet granule release upon reperfusion is probably minimal and as such there is no evidence for platelet activation upon reperfusion.

Although platelet activation leading to degranulation was not observed in the reperfused kidney, more delicate changes in the excitability of platelets could potentially be sufficient to change platelet homeostasis. We used a novel and sensitive bedside test to determine platelet excitability. Results showed that platelet excitability decreased when blood passed through the reperfused graft. This inhibitory effect on platelets passing through the reperfused kidney may be explained very well by an endothelial regulatory release of short-acting inhibitors of platelet activation, such as nitric oxide and prostacyclin.[26-28] Release of these factors unfortunately is difficult to confirm due to their short half-life. The difference is probably not mediated by adherence of platelets in the kidney, since platelet concentration was equal in arterial and venous blood, and immunohistochemical analysis did not reveal renal platelet deposits after reperfusion. Moreover, one would then expect a measurable release of platelet activation markers as well.

A limitation of our study was the fact that the sampling time was restricted to a maximum of 30 minutes following reperfusion. Although platelet activation is considered to be a short-term process, thought to occur during the first contact between recipient blood and the graft, we cannot exclude that platelet activation commences after this time period. Hence, apart from their potential role at later time points, platelets do not appear to be involved in the primary initiation of I/R injury. One may consider the sample size a limitation, but as

the goal of this study was to assess the basic pathophysiological role of platelet activation in I/R injury, instead of correlating findings to clinical outcomes, small patient numbers were sufficient.

In conclusion, we unequivocally show absence of platelet activation in early reperfusion injury in both living and deceased donor kidney transplantation. In fact, platelets display inhibited excitability upon passing the reperfused kidney. This indicates that platelets do not initiate the inflammatory response of I/R injury and other factors are more important in regulating the immune response.

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