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Microvascular alterations in transplantation

Khairoun, M.

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Author: Khairoun, Meriem

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Chapter 3

Renal ischemia/reperfusion induces release of angiotensin-2 from human grafts of living and deceased donors

Dorottya K. de Vries, Meriem Khairoun, Jan H.N. Lindeman, Ingeborg M. Bajema,
Emile de Heer, Mark Roest, Anton J. van Zonneveld, Cees van Kooten, Ton J. Rabelink,
Alexander F. Schaapherder, Marlies E.J. Reinders

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Abstract

Background: Recent insights suggest that endothelial cell (EC) activation plays a major role in renal ischemia/reperfusion (I/R) injury. Interactions between ECs and pericytes via signaling molecules, including angiopoietins, are involved in maintenance of the vascular integrity. Experimental data have shown that enhancement of Angiopoietin (Ang)-1 signaling might be beneficial in renal I/R injury. However, little is known about the role of angiopoietins in human renal I/R injury.

Methods: In this study, EC activation and changes in angiopoietins are assessed in human living and deceased donor kidney transplantation. Local release of angiopoietins was measured by unique, dynamic arteriovenous measurements over the reperfused kidney.

Results: Renal I/R is associated with acute EC activation shown by a vast Ang-2 release from both living and deceased donors shortly after reperfusion. Its counterpart Ang-1 was not released. Histological analysis of kidney biopsies showed EC loss after reperfusion. Baseline protein and mRNA Ang-1 expression was significantly reduced in deceased compared to living donors and declined further after reperfusion.

Conclusions: Human renal I/R injury induces EC activation after reperfusion reflected by Ang-2 release from the kidney. Interventions aimed at maintenance of vascular integrity by modulating angiopoietin signaling may be promising in human clinical kidney transplantation.

Introduction

Ischemia reperfusion (I/R) injury is an inevitable consequence of organ transplantation and a major determinant of patient and graft survival (1,2). The pathophysiology of renal I/R injury is complex and incompletely understood. Although the role of tubular cell injury in post-transplantation graft dysfunction is widely acknowledged, microvascular endothelial cell (EC) damage is considered increasingly important (3). ECs line the lumen of all blood vessels within the kidney graft and in this unique position they form the interface between the recipient blood and the allograft tissue. ECs are very susceptible to damage, including I/R injury (4). The repetitive insults during transplantation may induce loss of the microvasculature, on the long term resulting in impaired delivery of oxygen and nutrients to renal tubular epithelial cells, chronic ischemia and cell death (4-8).

The molecular mechanisms that lead to microvascular graft injury are largely unknown. Endothelial homeostasis is regulated by the angiotensin system and pericytes (9). Pericytes are the basic supportive cells of the endothelium, connected to the vessel's basement membrane (10). Angiotensin-1 (Ang-1) and -2 (Ang-2) are both ligands for the Tie2 receptor, but have opposite effects. Ang-1 is produced by pericytes and is responsible for suppressing vascular leakage, maintaining EC survival and inhibiting vascular inflammation. Ang-2 acts as an antagonist of Ang-1 and thereby destabilizes quiescent endothelium (11-14). Ang-2 promotes pericyte loss, leading to loosening contacts between ECs and pericytes, subsequent vessel destabilization and abnormal microvascular remodeling (15-18). ECs store Ang-2 in Weibel-Palade bodies from where it can be released quickly following stimulation, and Ang-2 expression can be upregulated manifold following endothelial activation (19). A few experimental studies have focused on the functional role of angiotensins in endothelial damage in the kidney. The relation between a disbalance in angiotensins and EC loss was demonstrated in a mouse model of anti-glomerular basement membrane glomerulonephritis, where glomerular capillary loss was associated with reduced Ang-1 and increased Ang-2 expression (20). Similarly, Ang-1 expression decreased after renal I/R injury in mice (21). Moreover, Ang-1 overexpression even significantly improved renal function and renal tissue blood flow after renal I/R in mice and decreases inflammatory cells and renal interstitial fibrosis (22).

Although these experimental data suggest a functional role of angiotensins in renal I/R injury, their involvement in human renal I/R injury had not been investigated yet. In addition, their involvement in living and deceased donor kidney transplantation had not been compared. In our recent studies on renal I/R, we exploited selective arteriovenous measurements over the kidney as a reliable method to study inflammatory processes after reperfusion (23,24). In this study, local renal angiotensin expression and release during renal I/R is systematically assessed in human living and deceased donor kidney transplantation.

Results

Integrity of the renal microvasculature is disturbed after reperfusion

To assess endothelial structure and viability, staining for ECs was quantified in both living and deceased donor kidney tissue. Both CD34 and vWf staining showed a characteristic positive pattern of peritubular and glomerular ECs. Pre-reperfusion biopsies did not show a difference between living and deceased donor kidneys in either CD34 ($p=0.76$) or vWf ($p=0.07$; Figure 1). Next, pre- and post-reperfusion biopsies of LD kidneys were compared. There was a trend towards a decrease in CD34 positive cells after reperfusion ($p=0.08$, data not shown), whereas vWf staining of ECs showed a significant decrease in expression after reperfusion ($p<0.001$; Figure 1).

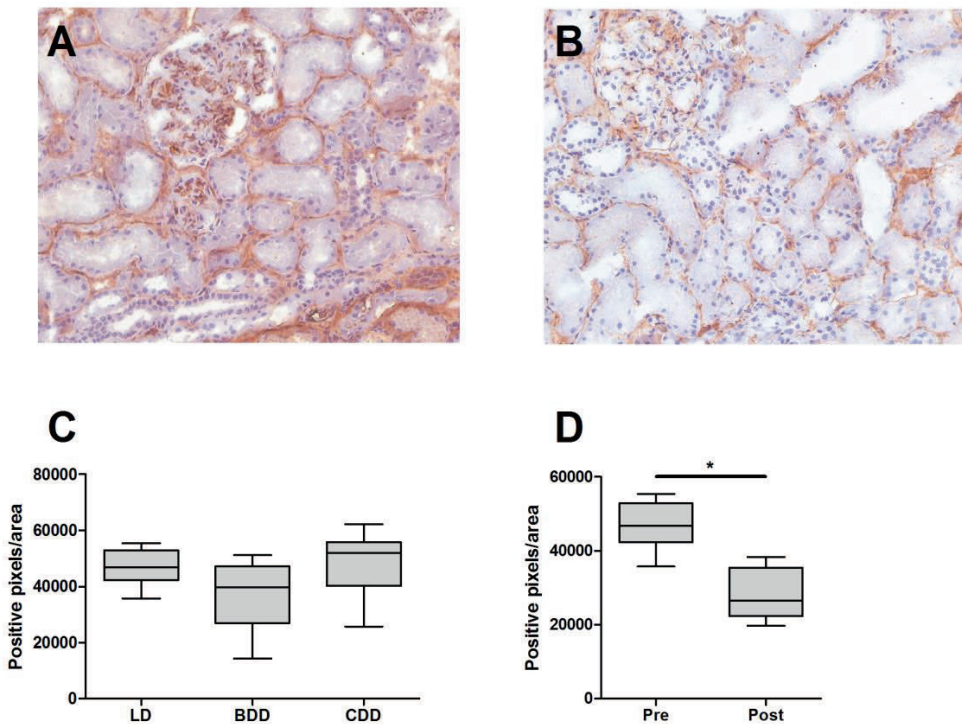


Figure 1: vWf staining decreased after reperfusion. Staining for vWf showed a characteristic positive endothelial pattern. Typical example of vWf staining in **A** a pre-reperfusion biopsy, **B** a post-reperfusion biopsy of a LD kidney. **C** Quantification of vWf staining showed no differences in intensity between groups before transplantation ($p=0.07$). **D** After reperfusion, vWf signal showed a vast decrease ($p<0.005$) in LD kidneys. The boxes run from the lower to upper quartile, the whiskers indicate the 5 to 95 percentile, while the horizontal line is set at the median.

Ang-2 is released from the reperfused kidney

Arteriovenous differences for Ang-1 and Ang-2 were assessed over the kidney during reperfusion. Ang-1 levels did not change significantly over the reperfused kidney in LD, BDD and CDD kidney transplantation, making it unlikely that anti-inflammatory Ang-1 is released from the kidney after reperfusion (Figure 2A-C). In contrast Ang-2, a marker for endothelial activation, was released from the kidney both early and late after reperfusion (Figure 2D-F). Ang-2 release was statistically significant in LD kidneys early after reperfusion (3 min., $p=0.005$; 30 min. $p=0.064$), in BDD kidneys only late after reperfusion (3 min. $p=0.15$; 30 min., $p=0.036$) and in CDD kidneys both early and late after reperfusion (3 min., $p<0.001$; 30 min., $p=0.017$). In the total group of deceased donor kidney transplantations, both early and late Ang-2 release was statistically significant (3 min., $p=0.024$; 30 min., $p=0.001$). The Ang-1/Ang-2 ratio was consistently higher in the arterial compared to renal venous samples at both time points for all donor types, although this trend was statistically not significant (data not shown). There was no early or late release of vWf or its more dynamic propeptide (Supplemental figure 1).

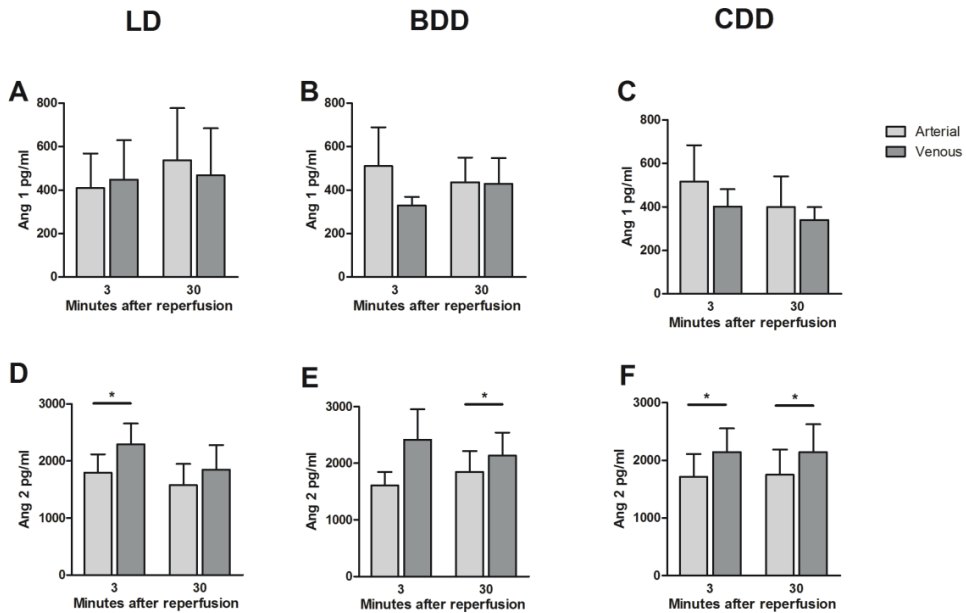


Figure 2: Ang-2 is released from the kidney during reperfusion. Ang-1 was not released from the kidney either 3 or 30 minutes after reperfusion in **A** LD (3 min. $p=0.18$; 30 min. $p=0.59$); **B** BDD (3 min. $p=0.29$, 30 min. $p=1.0$); **C** CDD (3 min. $p=0.47$; 30 min. $p=1.0$). Ang-2 was significantly released from the kidney in **D** LD kidneys early after reperfusion (3 min. $p=0.005$; 30 min. $p=0.064$), **E** in BDD kidneys only later after reperfusion (3 min. $p=0.15$; 30 min. $p=0.036$) and **F** in CDD kidneys both early and late after reperfusion (3 min. $p<0.001$; 30 min. $p=0.017$). $N=6$ in each group. Bars represent mean and error bars represent SEM.

Ang-1 protein and mRNA expression are significantly higher in LD kidneys and are reduced after reperfusion

Protein and mRNA expression of angiopoietins was assessed in the three donor groups. Ang-1 protein was expressed in glomeruli and capillary walls (Figure 3A, B). The protein expression was already reduced before transplantation in CDD kidneys ($p=0.003$), not BDD kidneys ($p=0.17$) compared to LD kidneys (Figure 3C, D). Consistently, Ang-1 mRNA expression before transplantation was significantly higher in LD kidneys compared to CDD kidneys ($p=0.006$) (Figure 3E). In LD kidney biopsies collected before and 45 minutes after reperfusion, there was a significant reduction in both Ang-1 mRNA ($p=0.007$) and protein expression after reperfusion ($p=0.001$; Figure 3D, F).

Ang-2 protein and RNA expression levels are similar between LD and deceased donors

Ang-2 immunostaining of kidney sections revealed expression in interstitial vessels and negative staining in the glomeruli (Figure 4A, B). There were no differences in Ang-2 protein and mRNA expression between LD, BDD and CDD kidneys ($p=0.69$, $p=0.73$, respectively; Figure 4C, E). Quantification of Ang-2 staining showed a decrease of protein expression after reperfusion in LD kidneys ($p=0.006$, Figure 4D). However, mRNA expression of Ang-2 did not change after reperfusion in LD kidney transplantation ($p=0.72$, Figure 4F). The mRNA expression of the angiopoietin receptor Tie-2 was similar between the different donor types ($p=0.48$) and not upregulated after reperfusion in LD grafts ($p=0.32$) (data not shown).

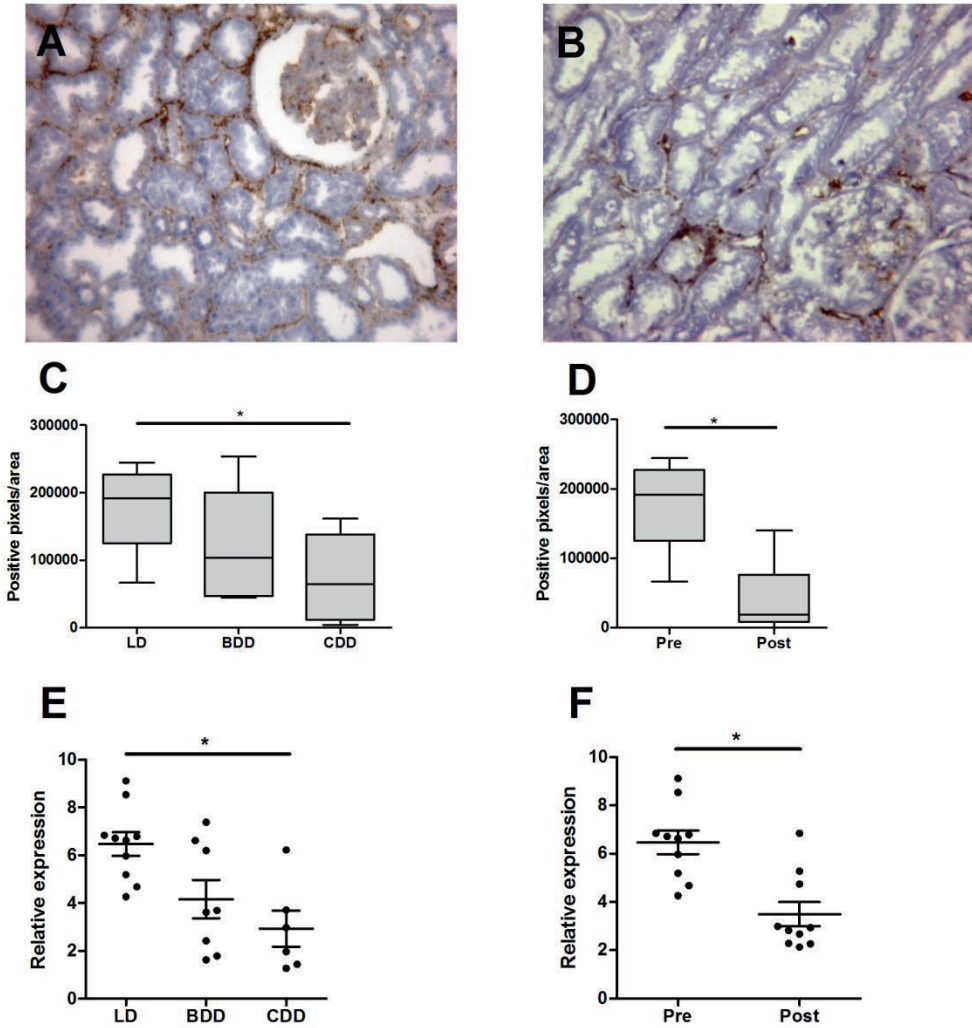


Figure 3: Ang-1 protein and mRNA expression decreased after reperfusion. Ang-1 staining was positive in glomeruli and peritubular capillaries. Typical example of Ang-1 staining in **A** a pre-reperfusion biopsy and **B** a post reperfusion biopsy of a LD kidney. **C** Quantification of Ang-1 in pre-transplantation kidney biopsies showed a significantly decrease in CDD ($p=0.003$) compared to LD grafts. **D** Ang-1 in LD kidney biopsies taken before (pre) and after (post) reperfusion showed a significant decrease in Ang-1 protein expression ($p=0.001$). The boxes run from the lower to upper quartile, the whiskers indicate the 5 to 95 percentile, while the horizontal line is set at the median. In kidney biopsies mRNA expression of Ang-1 was assessed. **E** pre-transplantation Ang-1 mRNA expression was significantly reduced in CDD kidneys compared to LD kidneys ($p=0.006$). **F** when comparing biopsies collected before (pre) and after (post) reperfusion, in LD kidneys there was a significant reduction in Ang-1 mRNA expression after reperfusion ($p=0.007$).

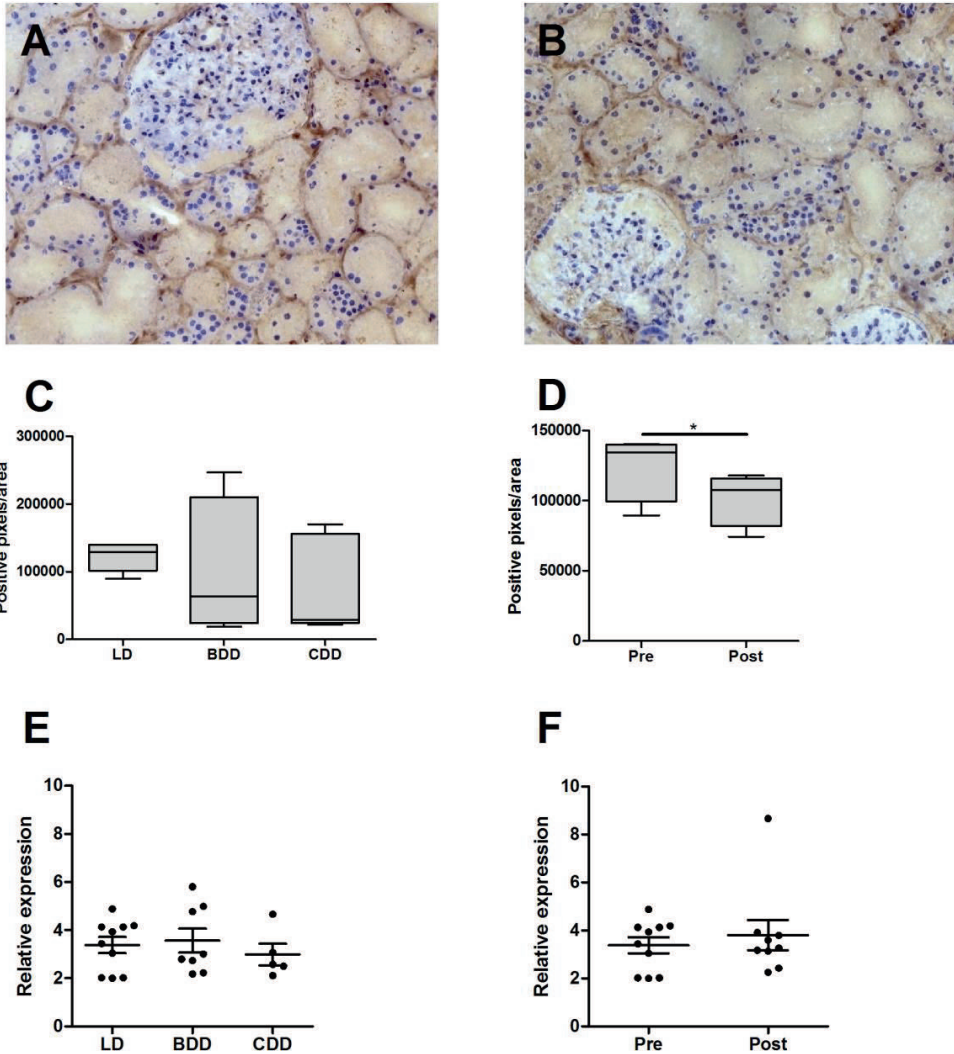


Figure 4: Ang-2 protein and mRNA levels are similar between living and deceased donors.

Ang-2 staining was positive in interstitial vessels and peritubular capillaries. Typical example of Ang-2 staining in **A** a pre-reperfusion biopsy and **B** a post-reperfusion biopsy of a LD kidney. **C** There were no differences in Ang-2 protein expression between groups before transplantation ($p=0.69$). **D** After reperfusion, there was a decrease in Ang-2 protein expression ($p=0.006$), consistent with degranulation of ECs and loss of Ang-2. The boxes run from the lower to upper quartile, the whiskers indicate the 5 to 95 percentile, while the horizontal line is set at the median. mRNA expression of Ang-2 was assessed in kidney biopsies. **E** Ang-2 expression was similar before reperfusion in all groups ($p=0.73$). **F** In LD kidney biopsies collected before (pre) and after (post) reperfusion, Ang-2 expression did not change ($p=0.721$).

Discussion

Previous studies, primarily exploiting animal models, have revealed that EC activation plays a major role in I/R injury and that the angiopoietin balance may be critical in maintaining vascular integrity. Enhancement of Ang-1 signaling has been demonstrated to be beneficial in renal I/R injury (25). However, thus far, no study has reported on the role of angiopoietins in human renal I/R injury or local release of angiopoietins from the kidney. In this study, the local response of the reperfused kidney was assessed by arteriovenous concentration differences of angiopoietins during the first 30 minutes after reperfusion in living and deceased donor kidney transplantation. Results demonstrate that renal I/R is associated with acute EC activation shown by a vast Ang-2 release from both living and deceased donor kidneys shortly after reperfusion.

In the present study, a reduction in CD34 and vWf protein expression was observed in tissue biopsies after reperfusion, consistent with either EC death or endothelial injury that induces loss of their characteristic epitopes. These findings are consistent with experimental models of acute ischemic renal endothelial injury after reperfusion (7,26,27), and recently observed in humans as well (28-31). EC activation represents a switch from a quiescent phenotype toward one that is involved in the host defense response. This process will result in expression of chemokines, cytokines, angiogenic factors and adhesion molecules designed to interact with leukocytes and platelets and target inflammation. In our previous study in human living donor kidney transplantation no evidence for EC activation was found by absence of local release of ICAM-1, P-selectin and vWf (23). However, the angiopoietins and endothelial/pericyte interactions were not studied.

Angiopietins have been shown to play a critical role in the maintenance of the microvasculature (15). This study focused on the release of these factors from both living and deceased donor kidneys. Results show that Ang-1 was not released from the kidney during reperfusion in any of the three groups, consistent with the theory that Ang-1 is constitutively expressed and dynamic changes in Tie-2 signaling are mediated via Ang-2. Consistent with previous reports, Ang-1 protein was found in glomeruli and capillary walls (32). Interestingly, we show that Ang-1 mRNA and protein expression decreased after reperfusion in human renal I/R injury. In contrast, in rodents Ang-1 expression has been shown to increase after chemical or ischemic kidney injury (33,34). However, these observations in experimental animals were done days after the injury, in contrast to the immediate changes observed in our patients. The loss of Ang-1 signal may indicate loss of vital pericytes, since pericytes are the main Ang-1 producing cells. However, decreased Ang-1 protein in renal tissue may also be related to the decrease in Ang-2 signal. Since Ang-1 and Ang-2 share a 60% amino acid identity, the storage

and release of Ang-1 may have the same mechanism as that of Ang-2 (35). Loss of Ang-1 signaling appears important in many disease processes, and enhancement of Ang-1 signaling was beneficial in sepsis and renal I/R injury (25,36).

High Ang-2 levels have repeatedly been shown in pathological conditions, such as in diabetes mellitus, renal failure and in myocardial I/R injury (37-39). Ang-2 triggers an inflammatory response by inducing permeability of the vascular lining (40), possibly mediated by the Ang-2 induced loss of pericytes (41). Results of this study show a vast Ang-2 release from both living and deceased donor kidneys shortly after reperfusion. This indicates injury to ECs, which can release Ang-2 from Weibel-Palade bodies upon activation (42). Since expression of Ang-2 did not change upon reperfusion, the arteriovenous difference may reflect Ang-2 release from Weibel-Palade bodies upon stress. This was confirmed by decreased endothelial Ang-2 tissue biopsy staining after reperfusion, consistent with exocytosis of Ang-2 containing granulae. In contrast to Ang-1, there was no expression of Ang-2 in the glomeruli, and positive signal was mainly seen on peritubular capillaries (32). The Ang-2 release from the kidney may induce continuing endothelial damage, ultimately leading to pericyte dropout, loosening contacts between endothelial and perivascular cells, with subsequent vessel destabilization and fibrosis.

In our arteriovenous measurements directly over the reperfused graft no release of vWf itself or its propeptide was observed, while Ang-2, another constituent of Weibel-Palade bodies was released. Possible explanations include that Ang-2 may be more easily detectable by arteriovenous measurements due to a shorter half-life, or that Ang-2 release from Weibel-Palade bodies is regulated independent from vWf release (43). Moreover, when vWf multimers are secreted from stimulated ECs they may remain anchored to the endothelial surface (44). This early retention of vWf to endothelium may provide a further explanation for the lack of a detectable release of vWf into the renal vein.

A limitation of our study was the fact that sampling time was restricted to maximally 45 minutes following reperfusion. Although endothelial damage is considered to be initiated during cold ischemia and expected to be vast around reperfusion, we cannot exclude continuing damage after 45 minutes. Another limitation is that we had to include different patient cohorts for arteriovenous measurements and collection of biopsies. Since for ethical reasons from every patient only one biopsy could be obtained, either DNA isolation or immunohistochemistry was done. Although one cohort for all sampling may have been preferable, our sampling is not expected to have influenced the conclusions from this study, since no correlations with patient characteristics are made. The aim of this study was to explore the endothelium-pericyte interactions in the pathophysiology of renal I/R injury. In future studies correlations between patient characteristics and angiotensin release will be made.

In conclusion, human renal I/R injury induces EC activation after reperfusion which leads to Ang-2 release from the kidney in both living and deceased donor kidney transplantation. This is accompanied by loss of ECs and diminished Ang-1 protein and mRNA expression. Moreover, compared to living donors expression of Ang-1 was significantly reduced in deceased donors. Interventions aimed at maintenance of vascular integrity by modulation of angiopoietin signaling may provide new therapeutical insights for I/R injury in human clinical kidney transplantation.

Materials and methods

Patient population

Eighteen patients undergoing renal allograft transplantation were included for arteriovenous sampling, 6 living donor (LD), 6 brain dead donor (BDD), and 6 cardiac dead donor (CDD) kidney recipients. BDD and CDD together are referred to as deceased donors (DD). Twenty-three other patients receiving a kidney from a LD (n=10), a BDD (n=7) or a CDD (n=6) were included for mRNA isolation from renal biopsies. Immunohistochemical analyses were performed on biopsies of 10 LD, BDD and CDD kidney graft recipients. Patient characteristics are shown in Supplemental Table 1. All kidneys were still functioning at 1 year after transplantation, except for one patient in the arteriovenous sampling cohort who received a kidney from a CDD (the recipient was not compliant with immunosuppressive medication) and one patient in the PCR cohort, that received a BDD kidney transplant and died 6 months after transplantation (because of veno-occlusive disease ultimately causing hepatic failure). For technical reasons (renal vein sampling) only patients receiving a left kidney were included in the arteriovenous group. The reason for including different cohorts was to minimize patient burden by collecting one type of material per patient, i.e. either blood or a single renal biopsy. The study protocol was approved by the local ethics committee, and informed consent was obtained from each patient.

Operation and materials

Kidney transplantations were performed according to local standardized protocol. In LD 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. DD kidneys were perfused and stored with University of Wisconsin solution (UW) or HTK. The immunosuppressive regimen was based on induction therapy with an interleukin-2 receptor blocker (basiliximab, day 0 and day 4) and maintenance treatment with tacrolimus or cyclosporine A, in addition to mycophenolate mofetil and steroids in all groups.

Arterial and renal venous blood samples were obtained as described before in detail (23). In short, via a small catheter placed in the renal vein, blood aliquots were sampled at 3, and 30 minutes after reperfusion. Paired arterial blood samples were obtained simultaneously. All samples were collected in tubes containing EDTA and immediately placed on ice. Blood samples were centrifuged (1,550 g, 20 min, 4°C) and the derived plasma was subsequently centrifuged to deplete it from leukocytes and platelets (1,550 g, 20 min, 4°C). Plasma was aliquotted and stored at -70°C until assayed.

Immunohistochemical analysis

A renal cortical biopsy was obtained after cold storage from LD, BDD and CDD kidney graft recipients. For logistical reasons, only in the LD group a second biopsy 45 minutes after reperfusion was collected as well. Immunohistochemical analyses were performed on 4 µm cryostat kidney tissue sections fixed in acetone. Slides were washed and incubated with peroxidase blocking solution and blocked with PBS containing 5% normal human serum (NHS) and 1% BSA. Then slides were incubated with the following primary antibodies: Ang-1 mouse monoclonal IgG, 1:800 (R&D systems), Ang-2 mouse monoclonal IgG, 1:100 (Novus Biologicals), ECs (CD34) mouse polyclonal IgG, 1:3200 (Dako), vWf, rabbit polyclonal, 1:2500 (Dako). Next, the following peroxidase-conjugated secondary antibodies were applied: goat anti-rabbit IgG, 1:200 (Dako); goat anti-mouse IgG, 1:200 (Dako); or rabbit anti-goat IgG, 1:200 (Dako), followed by sequential fluorescein isothiocyanate (FITC, amplification reagent) and anti-fluorescein-HRP. Staining was completed by incubation with 3,3' diaminobenzidine tetrahydrochloride (DAB)/ hydrogen peroxide and counterstained with Mayer's Hematoxylin Solution (Merck, Darmstadt, Germany). At 100 × magnification, 10 microscopic fields of each kidney section were quantified using computerized image analysis (ImageJ).

Plasma measurements of endothelial cell activation

Activation of the endothelium was assessed by measuring the local release of endothelial activation markers from the kidney. Release of Ang-1 and Ang-2 from the kidney was assessed by ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. The paired arteriovenous samples were also analyzed on more classical markers of endothelial activation; von Willebrand factor (vWf) and vWf propeptide. The propeptide has a shorter circulating half-time (2–3 h), compared with vWf itself (>12 h), and may therefore be a more sensitive marker of acute endothelial activation. Plasma levels of vWf and vWf propeptide were determined using a semi-automated ELISA. Plates were coated overnight with coating buffer, consisting of sodium carbonate, sodium bicarbonate and NaN₃. Commercial antibody duo sets, optimized for ELISA, were used (rabbit anti-human vWf and peroxidase-conjugated rabbit anti-human vWf, A00082 and P0226, Dako, Glostrup, Denmark). vWf propeptide, rabbit-anti-propeptide, and rabbit-anti-propeptide-biotine were used for the analysis of vWf propeptide as described previously (45).

mRNA expression in human kidney biopsies

A renal cortical biopsy was obtained after cold storage from LD, BDD and CDD kidneys, and a second biopsy was collected 45 minutes after reperfusion from LD kidneys. Biopsies were immediately snap frozen in liquid nitrogen and stored at -70°C. Total RNA was extracted from renal tissues using RNazol (Campro Scientific, Veenendaal, The Netherlands) and glass beads (46). The integrity of each RNA sample was examined by Agilent Lab-on-a-chip technology using the RNA 6000 Nano LabChip kit and a bioanalyzer 2100 (Agilent Technologies, Amstelveen, The Netherlands). RNA preparations were considered suitable for further processing if samples showed intact 18S and 28S rRNA bands, and displayed no chromosomal peaks or RNA degradation products (RNA Integrity Number >8.0) (47). cDNA was synthesized from 1 µg total RNA, using an oligo dT primer, RNase-OUT, M-MLV reverse transcriptase, 0.1 M-DTT and buffers in a volume of 20 µL (all purchased from Invitrogen, Breda, The Netherlands). Quantitative real-time PCR was performed in duplicate by using iQ SYBR Green Supermix on iCycler Real-Time Detection System (BioRad). The amplification reaction volume was 20 µL, consisting of 10 µL iQ SYBR Green PCR mastermix, 1 µL primers, 1 µL cDNA, and 8 µL water. Data were analyzed using Gene Expression Analysis for iCycler Real-Time PCR Detection System (BioRad). Expression of each gene was normalized against mRNA expression of the housekeeping gene GAPDH. The primer sequences are shown in Supplemental Table 2.

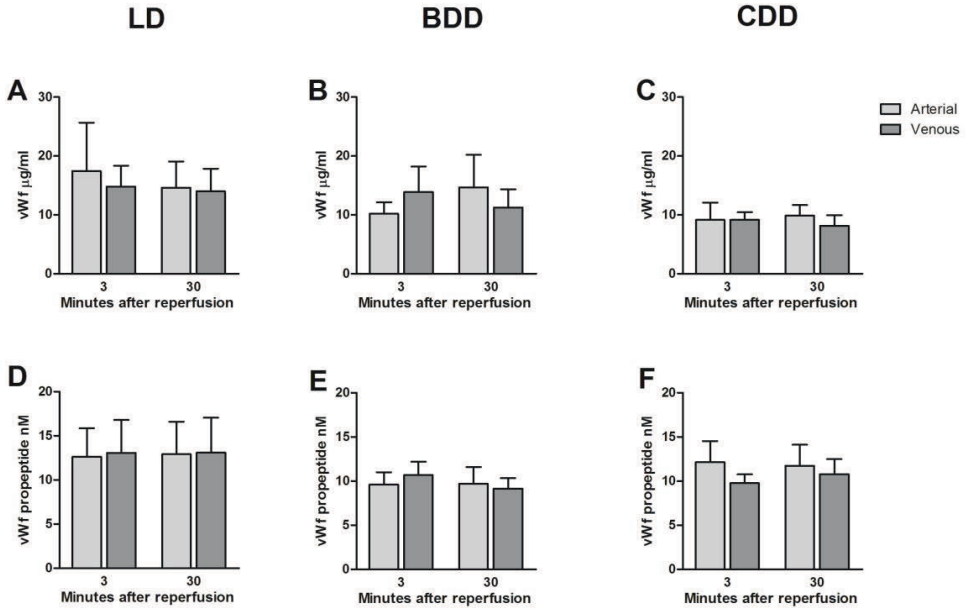
Analysis

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. Acute rejection was defined as biopsy proven rejection within 60 days after transplantation. Statistical analysis was performed using SPSS 17.0 statistical analysis software (SPSS Inc, Chicago, Ill). Paired, non-parametric test (Wilcoxon) was applied to assess differences between arterial and venous concentration, and between pre- and post-reperfusion samples. Graph error bars represent the standard error of the mean (SEM), unless otherwise stated. A *P*-value of less than 0.05 was considered significant.

Acknowledgements

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Supplemental material



Supplemental figure 1: No vWf or vWf propeptide release from the reperfused kidney. vWf was not released from the reperfused graft both early (3 min.) and later (30 min.) after reperfusion in kidney transplantation with **A** LD ($p=0.89$, $p=0.78$), **B** BDD ($p=0.89$, $p=0.33$) and **C** CDD ($p=0.40$, $p=0.18$) respectively. The shorter lived and more dynamic vWf propeptide, was not released either from the kidney in **D** LD ($p=0.78$, $p=0.62$), **E** BDD ($p=0.19$, $p=0.89$) and **F** CDD ($p=0.35$, $p=0.74$) kidney transplantation. Bars represent mean and error bars represent SEM.

Supplemental table 1: Transplantation and outcome characteristics of the three different patient cohorts that were included. In each cohort material was collected from living donor (LD), brain dead donor (BDD) and cardiac dead donor (CDD) grafts during kidney transplantation.

	AV			IHC			PCR		
	LD	BDD	CDD	LD	BDD	CDD	LD	BDD	CDD
N	6	6	6	10	10	10	10	7	6
Donor age: mean (SD)	42(11)	51(21)	52(16)	57(10)	48(16)	41(10)	52(8)	45(21)	41(13)
Donor gender (M:F)	5:1	2:4	3:3	5:5	5:5	6:4	2:8	3:4	4:2
WIT1 in min. (SD)	N/A	N/A	23(8)	N/A	N/A	18(5)	N/A	N/A	18(6)
CIT in h. (SD)	2.9(0.3)	21.3(7.1)	17.1(2.9)	2.8(0.5)	18.6(9.6)	17.8(3.4)	3.0(0.4)	17.4(7.6)	15.6(3.9)
WIT2 in min. (SD)	33(7)	31(6)	34(7)	29(8)	28(4)	28(8)	29(7)	34(3)	28(5)
Recipient age: mean (SD)	41(11)	57(13)	52(10)	54(16)	51(13)	45(15)	49(16)	53(14)	54(9)
Recipient gender (M:F)	3:3	2:4	5:1	4:6	7:3	7:3	6:4	4:3	3:3
Creatinine clearance day 30 (ml/min)	73(19)	64(13)	35(12)	45(11)	63(30)	48(21)	56(17)	47(14)	50(8)
DGF	0/6	3/6	5/6	0/10	3/10	7/10	0/10	3/7	4/6
DGF: dialysis after transplantation in days (SD)	0	5(6)	13(10)	0	5(2)	13(6)	0	3(4)	7(6)
Acute rejection n (%)	1(17%)	0(0%)	1(17%)	0(0%)	2(20%)	1(10%)	1(10%)	0(0%)	0(0%)

AV: cohort of patients included for arteriovenous sampling over the kidney; IHC: cohort of patients in which renal biopsies were used for immunohistochemical analysis; PCR: cohort of patients from which renal biopsies were used for PCR analysis; WIT1: first warm ischemia time; CIT: cold ischemia time; WIT2: second warm ischemia time; DGF: delayed graft function.

Supplemental table 2: Primer sequences used for quantitative real-time polymerase chain reaction.

Gene	Forward primer 5'->3'	Reverse primer 5'->3'	Supplier
GAPDH	TTCCAGGAGCGAGATCCCT	CACCCATGACGAACATGGG	Biolegio
Ang-1	GCAACTGGAGCTGATGGACACA	CATCTGCACAGTCTCTAAATGGT	Biolegio
Ang-2	TGGGATTTGGTAACCCTTCA	GTAAGCCTCATTCCCTTCCC	Biolegio
Tie-2	GTATGGA CTCTTAGCCGGCTT	TTCGCCCATCTCTGGTCAC	Biolegio

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