Angiopoietins in renal replacement therapy

Westendorp, Welmoet Hillegonda

CHAPTER 9

Summary, discussion and future perspectives

In patients with end-stage renal disease (ESRD), renal transplantation is the preferred therapy with enhanced patient survival compared to dialysis therapy. In renal transplantation, kidneys derived from living donors have better graft survival compared to kidneys obtained from deceased donors[1,2]. In order to completely utilize the donor pool, interventions improving transplant outcome, for instance by diminishing brain death-induced inflammation, are warranted. The demonstrated role of the endothelial Ang/Tie2-system in sepsis and endotoxemia made us question how this system would relate in the deceased brain death donor and subsequently other aspects of renal transplantation. We hypothesized that the Ang/Tie2-system plays a critical role in endothelial activation before and throughout renal transplantation, thereby affecting donor organ quality and patient and allograft outcome.

To study whether changes in angiopoietin expression were already present in the pre-transplant setting, we first studied plasma Ang1 and Ang2 in diaysis patients. In **chapter 2**, we show Ang1 and Ang2 levels in a well-defined cohort of 100 patients on hemodialysis. A marked peak of Ang2 levels was seen in these patients after 60 minutes of hemodialysis. Furthermore, significant associations between Ang2 and markers of inflammation, fluid overload and cardiac damage were found. In contrast no association was found between Ang1 and clinical parameters or dialysis outcome. Prospective analysis revealed that Ang2 levels are associated with a higher incidence of all-cause mortality and cardiovascular events. This extends previous research on Ang2 in dialysis, demonstrating that angiopoietin levels correlated with the presence and severity of coronary heart disease and peripheral arterial disease. Since CKD patients and patients on HD are characterized by endothelial dysfunction and endothelial dysfunction itself is a known cause of Ang2 release by Weibel Palade bodies, we speculate that the elevated predialysis Ang2 levels reflect endothelial activation. This is substantiated by the strong association between Ang2 and pro-endothelin levels that we found in these patients. These results underline the importance of understanding the responsible mechanisms concerning the Ang/Tie2-system. Elucidating this will provide the fundament for therapeutic intervention studies on the Ang/Tie2-system in hemodialysis.

Although circulating angiopoietin levels have been studied in renal transplant recipients and small numbers of living and deceased kidney donors[3-6], little is known of the angiopoietin response in the complete renal transplantation cascade, from donor to its paired recipient. Since is has become evident that Ang2 is the most rapid responder to pathologic circumstances and the fact that it seems to play a predictive role in various conditions, we studied plasma levels and arteriovenous renal Ang2 release in living donor renal transplantation in **chapter 3**. Both in donors and recipients, plasma Ang2 changed during the operation compared to the preoperative levels while reperfusion did not affect renal Ang2 release. In contrast, another study on renal Ang2 release in living and DBD transplantation

demonstrated increased renal Ang2 release of the reperfused kidney grafts in living and DBD transplantation. The use of the same Ang2 ELISA in that study makes a technical cause of the difference in results between our studies unlikely. Perhaps both living donor populations are not comparable when it comes to the degree of endothelial activation before and during nephrectomy. Additional multi-center Ang2 analysis in renal transplantation are required to further shed light on the intracentral differences in living donor selection and endothelial activation during retrieval.

In **chapter 4** we describe Ang1 and Ang2 levels in renal transplant recipients (RTR) and their controls. In line with previous studies by our group and others, Ang2 associated with markers of inflammation and cardiac damage. This is consistent with previous data in which Ang2 levels are positively associated with CRP in another cohort of RTR[4]. Our results showed higher plasma Ang2 in RTR who received a kidney from a deceased donor compared to those RTR who received a kidney from a living donor. In these deceased donor-RTR, Ang2 was associated with graft failure and mortality. Possibly RTR suffer a pre-inflammatory state which aggravates in case there is a risk for organ failure, reflected by increased Ang2-supported endothelial activation[7-12,15,16].

Deceased brain dead (DBD) donors traditionally have been the main source of donor organs in renal transplantation. The results of further studying the histopathological characteristics of the morphological inflammatory responses caused by brain death in (pre-) transplant renal biopsies are described in **chapter 5**. Although our group and others have demonstrated the detrimental effect of BD on organ quality and graft survival, it is still difficult to predict donor organ quality at time of donor management and evaluation. Early pre-transplant predictions of transplant outcome by numerous histopathological damage parameters and scoring systems have been studied. These results have however not led to a unanimous approach[13-16]. In addition to the classical damage parameters, we were particularly interested in lymph vessel density (LVD). Although the importance of LVD in the development and progression of renal pathophysiology -and transplantation has been convincingly shown, it is not well studied in pre-transplant biopsies and BD. As anticipated, pretransplant biopsies of DBD donors showed increased LVD and pro-fibrotic, vascular and inflammatory damage compared to the biopsies obtained from living donors. Within the DBD group, LVD was correlated with signs of arteriopathy such as hyalinosis and with markers of interstitial injury such as interstitial fibrosis. Whether the increase in LVD is the cause or the effect of vascular damage is unknown, but it might well be that pathologic thickening of the vascular wall hampers normal vascular function including fluid transport, thereby resulting in increments in LVD. Validation of LVD in the assessment of kidney quality before transplantation and evaluation whether a separate scoring approach is required for DBD donors needs to be determined in prospective analysis.

In **chapter 6** deceased donor and recipient polymorphisms in the Ang2 gene were determined in a large renal transplant cohort. Associations between Ang2 single nucleotide polymorphisms (SNPs) and the development of acute lung injury and increased risk of acute respiratory distress syndrome have been reported. These findings together with the possibility of changed Ang2 protein expression and effect among different Ang2 SNPs, and our suggested role of Ang2 in deceased donation and RTR, resulted in a genetic renal transplant cohort Ang2 study. Tagging Ang2 SNPs were associated with death censored graft survival when analyzed in both donor and recipient. No association was found with all-cause mortality. To confirm the role of Ang2, the functionality of these Ang2 SNPs has to be confirmed by replication studies.

In **chapter 7** we reviewed the literature on brain death (BD) and presented an overview of the current knowledge of renal injury caused by BD. We described the current knowledge during this unphysiological state, its effect on kidney organ quality, potential mechanisms of repair and its relevance for renal transplant outcome. Intracranial hemorrhage or traumatic brain injury are usually the cause of brain compression leading to irreversible cerebral injury. Ultimately the brain stem herniates with loss of brain stem reflexes, a Cushing reflex due to catecholamine release followed by hypotension and finally complete circulatory arrest. Due to complete dysfunction of the hypothalamo-pituitary axis most patients suffer from diabetes insipidus. Shortly after the onset of brain death, high levels of circulating proinflammatory cytokines such as interleukin-6 can be detected. BD results in endothelial activation and increased influx of polymorphonuclear neutrophils (PMNs) and macrophages in kidney, liver and intestine. The inflammatory response on organ level requires interventional strategies aiming at enhanced graft quality and subsequently better graft survival of kidneys obtained from DBD donors.

Frequent occurrence of endotoxemia has been shown in DBD donors[17]. Endotoxemia influences the Ang/Tie2-system by triggering an increased vascular permeability[18]. A disturbed Ang1/Ang2 ratio in favour of Ang2 is associated with increased mortality during sepsis, while a ratio in favour of Ang1 maintains vascular integrity and dampens the inflammatory response[19-21]. The known endotoxemic status together with the pathophysiological similarities between BD and sepsis makes the Ang/Tie2-system an interesting intervention target in aiming to counteract the detrimental consequences of BD. Experimental studies on recombinant and Ang1 derivates like recombinant human Ang1 (rhAng1) and Vasculotide (VT), have demonstrated promising results in murine sepsis and protecting adult vessels against plasma leakage[21-23]. Therefore, we studied Ang/Tie2-axis manipulation via two Ang1 enhancing strategies in our brain death rat model.

In **chapter 8** we describe the first proof of principle experiments aiming to modulate the Ang/Tie2-system by testing exogenous Ang1 administration and Ang2 inhibition in experimental brain death. BD rats were treated with

rhAng1 or a vehicle and compared with sham-operated rats. In a second experiment, BD rats were treated with VT or a vehicle. Unfortunately, neither rhAng1 nor VT treatment did protect against brain death induced inflammation in rat kidneys. This may be explained by a dosage or timing problem. Possibly Ang1 enhancing therapy may be more beneficial during BD itself, immediately competing with the increased Ang2 in order to bind Tie2. The dosages we used were based on previous sepsis experiments in mice. Even though we took the difference in metabolism and weight of the rats into account, the dosages may have been too low. We can therefore not exclude a potential anti-inflammatory effect when higher dosages are used. Real-time PCR analysis revealed a marked decrease in Tie2 mRNA expression in BD rats which could be another explanation why the Ang/Tie2-system was not beneficially affected by exogenous administration of these Ang1 derivates. Although our analysis made it indefinable whether the Ang/Tie2-system was actually modulated in favour of Ang1 by these dosages, the results on mRNA expression made this highly unlikely.

Although the majority of preclinical studies on similar pathophysiological conditions as BD have approached intervention strategies via the Ang/Tie2-system by upregulating Ang1, recent literature is more and more focussing on Ang2 as the most dynamic player in endothelium activated processes. Others have reported promising results on anti-Ang2 therapy in various preclinical models including a study on cardiac rat allografts. Therefore, in a third experiment we studied whether inhibiting Ang2 could attenuate BD induced renal inflammation in the rat model mimicking the DBD donor. This study was designed as a single dose pilot in which systemic Ang2 was not reduced by this treatment. We observed no reduction in systemic or renal inflammation in this group compared to the vehicle treated BD rats. Until now we and others have studied Ang/Tie2-intervention strategies approaching Ang1 or Ang2 levels. A dosing titrating study on exogenous Ang1 or an Ang2 inhibitor will learn us which dosage should be further studied. After this an experimental BD time course-effect study of Ang/Tie2-system intervention will give the opportunity to draw more definite conclusions. Although the results from our single-dose BD pilots are inconclusive on manipulating these levels in BD, Tie2 preserving strategies may be another interesting target in attenuating BD induced inflammation since we observed a profound reduction in Tie2 mRNA in renal tissue of BD rats and a trend in reduced Tie2 protein expression in BD mice. Before testing drugs that prevent Tie2 loss, the marked decrease in BD rat renal Tie2 expression needs to be confirmed in human DBD donors. Not only systemic donor pre-treatment but also blocking Ang2 induced processes via an Ang2 inhibitor or Ang1 derivate during the preservation period may be beneficial aiming to preserve/maintain organ viability/quality.

In conclusion, this thesis demonstrates that the Ang/Tie2-system is quite dynamic before, during and after renal transplantation, especially in the DBD donor. The endothelial activation in hemodialysis, kidney donor and transplant recipient may have been reflected by the altered angiopoietin levels. However, the altered angiopoietin activation pathway may participate in the further deterioration of endothelial function in dialysis and transplant patient thereby shifting this system from biomarker to causative factor in vascular damage. Overall our studies reveal that there is a need for further therapeutical intervention studies in the angiopoietin/Tie2 pathway in experimental and cell culture conditions. These may ultimately pave the way for human Ang/Tie2-therapy studies in dialysis and transplant recipients.

FUTURE PERSPECTIVES

The potential of measuring Ang2 as marker for organ quality at time of donation needs to be determined in a clinical study including living and deceased donors and assess its sensitivity and specificity versus other markers. Until then it remains unclear if the associations we describe would translate into one of clinical impact. Moreover, a quick, validated clinical applicable Ang2 test should be developed before clinical implementation would be possible. Furthermore, the predictive potential of Ang2 on transplant outcome should also be investigated in other organ transplants. Given the widely described dynamics of the Ang/Tie2-system and the possibility to manipulate its effect by several intervention strategies, angiopoietins may not only serve as predictive or quality markers, but also as intervention target. Specifically, elucidating the Ang/Tie2-mechanism and its role in BD is further study worth. Generating more understanding of the molecular mechanisms that affect the Ang/Tie2-system would support study design in humans to ultimately translate to the clinical setting. Especially in defining future therapeutic approaches to inhibit endothelial dysfunction via the Ang/Tie2-system in hemodialysis, the DBD donor and renal transplant recipients.

REFERENCES

1. Terasaki PI, Cecka JM, Gjertson DW, Takemoto S. High survival rates of kidney transplants from spousal and living unrelated donors. N Engl J Med. 1995;333(6):333-336.
2. Pratschke J, Wilhelm MJ, Kusaka M, et al. Accelerated rejection of renal allografts from brain-dead donors. Ann Surg. 2000;232(2):263-271.
3. Molnar MZ, Kumpers P, Kielstein JT, et al. Circulating angiopoietin-2 levels predict mortality in kidney transplant recipients: A 4-year prospective case-cohort study. Transpl Int. 2014.
4. David S, Kumpers P, Hellpap J, et al. Angiopoietin 2 and cardiovascular disease in dialysis and kidney transplantation. Am J Kidney Dis. 2009;53(5):770-778.
5. Dane MJ, Khairoun M, Lee DH, et al. Association of kidney function with changes in the endothelial surface layer. Clin J Am Soc Nephrol. 2014;9(4):698-704.
6. de Vries DK, Khairoun M, Lindeman JH, et al. Renal ischemia-reperfusion induces release of angiopoietin-2 from human grafts of living and deceased donors. Transplantation. 2013;96(3):282-289.
7. van de Berg PJ, Hoevenaars EC, Yong SL, et al. Circulating lymphocyte subsets in different clinical situations after renal transplantation. Immunology. 2012;136(2):198-207.
8. Vereyken EJ, Kraaij MD, Baan CC, et al. A shift towards pro-inflammatory CD16+ monocyte subsets with preserved cytokine production potential after kidney transplantation. PLoS One. 2013;8(7):e70152.
9. Damman J, Schuurs TA, Ploeg RJ, Seelen MA. Complement and renal transplantation: From donor to recipient. Transplantation. 2008;85(7):923-927.
10. Rabb H, O'Meara YM, Maderna P, Coleman P, Brady HR. Leukocytes, cell adhesion molecules and ischemic acute renal failure. Kidney Int. 1997;51(5):1463-1468.
11. Fiedler U, Reiss Y, Scharpfenecker M, et al. Angiopoietin-2 sensitizes endothelial cells to TNF-alpha and has a crucial role in the induction of inflammation. Nat Med. 2006;12(2):235-239.
12. van Meurs M, Kumpers P, Ligtenberg JJ, Meertens JH, Molema G, Zijlstra JG. Bench-to-bedside review: Angiopoietin signalling in critical illness - a future target? Crit Care. 2009;13(2):207.
13. Rule AD, Semret MH, Amer H, et al. Association of kidney function and metabolic risk factors with density of glomeruli on renal biopsy samples from living donors. Mayo Clin Proc. 2011;86(4):282-290.
14. De Vusser K, Lerut E, Kuypers D, et al. The predictive value of kidney allograft baseline biopsies for long-term graft survival. J Am Soc Nephrol. 2013;24(11):1913-1923.
15. Nickeleit V. Foretelling the future: Predicting graft outcome by evaluating kidney baseline transplant biopsies. J Am Soc Nephrol. 2013;24(11):1716-1719.
16. Nickeleit V. Pathology: Donor biopsy evaluation at time of renal grafting. Nat Rev Nephrol. 2009;5(5):249-251.
17. van Goor H, Rosman C, Grond J, Kooi K, Wubbels GH, Bleichrodt RP. Translocation of bacteria and endotoxin in organ donors. Arch Surg. 1994;129(10):1063-1066.
18. Kumpers P, van Meurs M, David S, et al. Time course of angiopoietin-2 release during experimental human endotoxemia and sepsis. Crit Care. 2009;13(3):R64.
19. van der Heijden M, van Nieuw Amerongen GP, Chedamni S, van Hinsbergh VW, Johan Groeneveld AB. The angiopoietin-Tie2 system as a therapeutic target in sepsis and acute lung injury. Expert Opin Ther Targets. 2009;13(1):39-53.

20. Parikh SM, Mammoto T, Schultz A, et al. Excess circulating angiopoietin-2 may contribute to pulmonary vascular leak in sepsis in humans. PLoS Med. 2006;3(3):e46.
21. David S, Park JK, Meurs M, et al. Acute administration of recombinant angiopoietin-1 ameliorates multiple-organ dysfunction syndrome and improves survival in murine sepsis. Cytokine. 2011;55(2):251-259.
22. Thurston G, Rudge JS, Ioffe E, et al. Angiopoietin-1 protects the adult vasculature against plasma leakage. Nat Med. 2000;6(4):460-463.
23. Kumpers P, Gueler F, David S, et al. The synthetic tie2 agonist peptide vasculotide protects against vascular leakage and reduces mortality in murine abdominal sepsis. Crit Care. 2011;15(5):R261.