The alloantigen-independent factors brain death and cold ischemia
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Chapter 1:

General Introduction and Aims of the Study
General Introduction

Causes for and Difficulties in Renal Transplantation

End-stage renal disease is the final state of an advanced restriction of exocrine-and endocrine functions of the kidney and makes survival of critically ill patients without adequate therapy impossible. A number of renal replacement therapies are available and include hemodialysis, peritoneal dialysis (CCPD and CAPD) and renal transplantation. Dialysis modalities can substitute, even in the most favourable case, for only approx. 10% of the exocrine renal function and for none of the endocrine functions [1]. In addition, it is time consuming and is a significant factor for the increase in health-care costs. More importantly, patients on dialysis have a significantly higher mortality rate and a lower quality of life [2-4]. Therefore renal transplantation is the best choice for patient with end-stage renal disease.

The first successful kidney transplantation was carried out in 1954 by Murray with monozygotic twins as donor and recipient [5, 6]. Nowadays, as modern immunosuppressive drugs have found their way in clinical practice, renal transplantation has become a standard therapy for patients with terminal renal failure [7]. Over the past decades, one year allograft survival has improved dramatically because of modern immunosuppression and improvements in pre-transplantation diagnostics. Yet, the rate of decline in long-term graft survival did not significantly change. Hence, chronic allograft loss remains a major hurdle in transplantation medicine [8]. Although for deceased donors the importance of HLA matching on graft survival has been demonstrated [9], more recently the significance of HLA matching has been challenged by the finding that poorly matched kidneys from living donors showed a better graft survival than kidneys from well matched deceased donors (3 – year survival rate 85% and 70% respectively) [10]. This argues against a dominant role for HLA-matching on graft survival and indicates that alloantigen-independent factors also must play a significant role in transplantation outcome. Living donors and deceased donors differ in two major alloantigen-independent factors, i.e. brain death and prolonged cold preservation.

There exist a multitude of risk-factors that are associated with graft loss. Acute and chronic graft rejection, nephrotoxicity due to calcineurin-inhibitors (CNI), cold ischemia time and recurrence of focal and segmental glomerulonephritis (FSGN) belong to the most important ones [11, 12].
Graft loss

Alloantigen-Dependent Factors

Graft rejection is a clear example of an alloantigen-dependent factor. It occurs as a consequence of immunity against donor antigens (e.g. MHC, blood group antigens, others) and is mediated by both humeral and cellular effector mechanisms. There exist three different types of rejection.

Hyperacute Rejection

Hyperacute rejection occurs within minutes after reperfusion, is antibody driven and is caused by circulating pre-existing anti-donor antibodies that can bind to blood group - or polymorphic MHC antigens on endothelial cells. Binding of these antibodies results in complement activation [13, 14], which in turn can activate the coagulation cascade [13]. Consequently, formation of intravascular thrombi can lead to vascular occlusion. Pre-existing anti-donor antibodies can be caused by blood transfusions, multiple pregnancies or previous transplantations. Due to improved pre-transplantation diagnostics, hyperacute rejection has become rare [15, 16], nevertheless it might occur in patients with anti-endothelial cell antibodies of unknown specificity [17].

Acute Rejection

There are two types of acute rejection, i.e. acute interstitial - and acute vascular rejection. Acute interstitial rejection is mediated by T-cells and is characterized in renal transplantation biopsies by tubular infiltration of leucocytes (tubulitis). T-cells, release of cytokines and recruitment of other immune-competent cells lead to renal injury within days to weeks. Because alloreactive T-cells are directed against foreign MHC molecules, this might explain the influence of HLA-matching on graft survival for deceased donors [18]. Although HLA identical grafts have a significantly better graft survival [19, 20], acute interstitial rejection nevertheless might occur. Serologically matched HLA identical grafts are not necessarily genetically identical and therefore small HLA differences could still exist. In addition, apart from the MHC loci, other polymorphic proteins may differ between donor and recipient and give rise to a cell mediated immune response. Alloantigens are presented to T-cells either by donor antigen-presenting cells (direct pathway) or by recipient antigen-presenting cells (indirect pathway) [21]. In the latter situation alloantigens must be processed by antigen-presenting cells of the recipient, and subsequently presented to T-cells as peptides in the context of recipient MHC-class-I or class II molecules.
The second type of acute rejection is acute vascular rejection. In contrast to the former type, it is believed that antibodies directed against endothelial cells are involved [13, 14, 22, 23]. Endothelial C4d deposition is an established histological marker for antibody-mediated acute vascular rejection [24, 25]. In addition to a humeral immune response acute vascular rejection might be mediated by a cellular immune response. The presence of vasculitis, i.e. leucocyte infiltration in the vessel wall, is also a hallmark for acute vascular rejection.

Acute interstitial rejection can be treated effectively by modern immunosuppressive drugs, while acute vascular rejection is still a clinical problem and is an important cause for early and late graft loss [26, 27].

**Chronic Rejection**

Chronic rejection is the leading cause for late graft loss. The aetiology of chronic rejection is not clear, but it is believed that inflammatory stimuli, e.g. cytokine release, recruitment of monocytes and macrophages are involved in the pathogenesis of this type of rejection [28]. Concentric atherosclerotic depositions in the blood vessels, accompanied by glomerular sclerosis and tubular atrophy are according to the Banff classification typical histological hallmarks for chronic allograft nephropathy [29-31].

Apart from alloantigen dependent factors, alloantigen independent factors might contribute to the development of chronic rejection. In this context preservation injury, donor brain death, CNI-toxicity, infection with cytomegaly virus are amongst others important alloantigen-independent factors [32-34].

**Alloantigen-Independent Factors**

**Ischemia-Reperfusion Injury and Cold Preservation**

During renal transplantation warm ischemia is unavoidable. The first warm ischemia time refers to the time from the beginning of clamping the arteria renalis up to the beginning of organ perfusion with cold preservation solution. This is followed by cold ischemia or cold storage, i.e. the time that the organ is in transit until the time of organ reperfusion in the recipient. The second warm ischemia time is the time required for anastomosis and is usually short.

Organ preservation is a pre-requisite for organ allocation and is mostly performed by cold storage. Nevertheless, prolonged cold storage leads to tissue injury due to an increased induction of apoptosis and/or necrosis [35-38]. Prolonged cold preservation also impairs the
endothelial barrier function resulting in parenchymal edema and hemorrhage [39]. This in turn, negatively affects early renal function and long-term graft survival [40].

Ischemia and subsequent organ reperfusion can result in strong structural changes, which may also cause delayed graft function [41]. This type of tissue injury mostly develops because of unspecific inflammatory reactions, reinforced leucocyte recruitment, activation of the endothelium paralleled by an increased expression of cytokines and adhesion molecules [42, 43]. An important role in the development of ischemia reperfusion injury is also credited to free oxygen radicals. Free oxygen radicals are produced during ischemia and reperfusion by an increased degradation of ATP to hypoxanthine [44, 45]. They damage cell membranes and macromolecules and can induce the release of inflammatory mediators and proteolytic enzymes [46, 47].

**Brain death**

Although brain death is well defined from a neurological point of view, less is known about the influence of brain death on peripheral inflammation in end-organs in mechanistic terms. In the majority of cases brain death is induced by trauma or intracranial bleeding. A rise in intracranial pressure with consecutive constriction of the brainstem and compression of venous and arterial vessels result in a complete loss of intracranial circulation and finally to coma [48]. Cerebral activity, spontaneous respiration and brainstem reflexes pause irreversible. Brain death is typically associated with a so called "autonomic storm", i.e. a marked increase in blood pressure as a consequence of vasoconstriction, followed by a sharp decline in blood pressure. Vasoconstriction is the result of a huge increase in serum catecholamine concentration (up to 1000-fold) within minutes [49]. Hemodynamic instability, hypothermia, coagulopathy and electrolyte abnormalities can occur consecutively [50]. Also other hormonal circuits are impaired. The hypothalamo-pituitary axis is often destroyed by brain death entailing the appearance of a central diabetes insipidus [50]. High serum catecholamine concentrations and decreased T 3-and cortisol levels result in an increased intracellular calcium concentration which entail an increased ATP consumption, subsequently leading to an inappropriate cellular energy supply [51].

In the past decades experimental evidence has indicated that brain death in organ donors enhances graft immunogenicity. This may also explain why long-term graft survival is better when renal grafts are retrieved from living donors as opposed to grafts from brain-dead donors, despite poor HLA-matching of the former [10]. A number of proinflammatory cytokines, e.g. IL-6 or TNF-α are increased in serum of brain-dead animals [52-54]. Also
gene expression studies of renal tissue from brain-dead animals showed a higher expression of cytokines, chemokines, selectins and fibrinogen, especially in hemodynamic instable animals [55]. Concomitantly to the increased expression of inflammatory molecules an increase in mononuclear cell infiltration have been reported [56, 57]. The group of Tilney [58] could show in an animal model that brain death induced infiltration of leucocytes, most likely via upregulation of adhesion molecules like ICAM-1, E-and P-selectins. They also found that the production of complement, interleukin-1beta and other proinflammatory chemokines and cytokines was increased in grafts of brain dead animals. Other studies have shown that brain death enhances ischemia-reperfusion injury and accelerates acute and chronic rejection in transplanted grafts [59, 60].

Strategies for graft protection

Demographic changes, an increased incidence of end-stage organ failure and a relative stable supply of donors are leading to an increasing gap between demand and supply of donor organs. In 2007 approximately 11,000 patients were waiting for kidney transplantation in Europe, but only around 3900 kidneys from deceased donors were available and only around 3400 transplantations were performed (Eurotransplant, annual report 2007). The increasing gap between demand and supply has forced transplant physicians not only to use organs obtained from optimal donors, but also organs from so-called “marginal donors”. These donors fulfil the extended criteria for organ donation, are in general older and might have previous diseases such as diabetes. Appropriate donor management is also of utmost importance in this context, as it may reduce the number of donor organs that are not accepted for transplantation because of organ damage.

The importance of donor pre-treatment is to maintain organ quality before implantation. Because organ quality is a risk factor for chronic organ loss, donor pre-treatment could positively affect the donor-recipient balance by increasing graft survival time. The use of organs from so called "marginal donors" can also effectively increase the potential donor pool.
Impairment of physiological control circuits in brain-dead organ donors

Hormonal resuscitation

In the acute phase of traumatic brain injury marked changes in the hypothalamo-pituitary axis have been documented [61-63]. As a consequence gonadotropin deficiency, growth hormone deficiency and corticotrophin deficiency could occur. Furthermore patients could show an evidence of thyroid hormone imbalances and inappropriate antidiuretic hormone secretion leading to diabetes insipidus or the syndrome of inappropriate anti-diuresis [64, 65]. Therefore fluid resuscitation, methylprednisolone bolus, liberal use of thyroid hormone and early use of vasopressors in hemodynamic unstable brain-dead donors is frequently applied at many intensive care units. Appropriate donor management increases the pool for organ donation and is associated with significant increases in organs transplanted per donor [66-68].

The Inflammatory Reflex

The question, why brain death negatively affects peripheral organs, can be answered only partially. Do organs sense the state of brain death, and if so, how this is accomplished, remains to be answered. Clearly changes in microcirculation and hormonal abnormalities could lead to damage in end-organs. This damage might give rise to an inflammatory process. There is unambiguous evidence that the parasympathetic nervous system can modulate innate immunity [69], [70, 71]. The vagus nerve innervates a variety of visceral organs and might be considered as the interface between the central nervous system and the immune system. In the cholinergic anti-inflammatory pathway, also referred as inflammatory reflex, the vagus nerve is of eminent importance [72]. Paraganglia cells located in the parasympathetic paraganglia express IL-1 receptors [73], which allows to signal the presence of peripheral inflammation to the brain. Subsequently, acetylcholine is released from the efferent nerve fibres as a negative feedback for control of excessive inflammation [71] (Figure 1). Acetylcholine, the principle neurotransmitter of the vagus nerve, controls immune cell functions via the alpha7 nicotinic acetylcholine receptor [74]. This results in inhibition of NF-κB activation and thereby controlling the production of proinflammatory cytokines [75, 76].
The parasympathetic anti-inflammatory pathway by which the brain modulates systemic inflammatory responses was first described by Borovikova et al. in a sepsis model [77]. Acetylcholine significantly attenuated the release of TNF\(\alpha\) and prevented the development of septic shock. In \textit{in vitro} and \textit{in vivo} models, cholinergic agonists also revealed an inhibitory effect on TNF\(\alpha\) induced endothelial cell activation. This was reflected by an inhibition in the expression of endothelial cell adhesion molecules in vitro and blockade of leukocyte migration in vivo in an air pouch model [78]. Vagus nerve stimulation in rats modulates the inflammatory response during acute hypovolemic hemorrhagic shock resulting in an improved survival [79]. Moreover, vagus nerve stimulation significantly attenuates TNF synthesis and shock during reperfusion injury in a standard model of aortic occlusion [80]. A number of studies already have shown a correlation between brain injury and autonomic neurologic dysfunction measured by heart rate variability in humans [81, 82]. Although there is no experimental evidence that brain death impairs the inflammatory reflex, it is conceivable that vagus nerve activity ceases in the course of brain death and this might therefore account for brain death induced inflammation. This will be discussed in chapter 2 of my thesis.

\textbf{Preconditioning of organ donors in renal transplantation}

The concept of donor pre-treatment is based on minimizing peri-operative organ damage by induction of cytoprotective mechanisms or by modulation of graft immunogenicity.

It has been shown that peri-operative treatment of brain-dead donor animals with steroids can significantly improve graft survival by inhibiting the expression of proinflammatory cytokines.
Similarly, inhibition of P-selectin-mediated leucocyte adhesion by the use of soluble P-selectin-glycoprotein-ligand (sPSGL) can improve long term graft survival [83].

**Dopamine in Transplantation Medicine**

From 1999 on we have focused on dopamine as a potential drug for preconditioning of deceased donors. Our interest started from the clinical observation that donor catecholamine use reduces acute allograft rejection and improves graft survival after cadaveric renal transplantation. Subsequent retrospective clinical studies also demonstrated the impact of donor dopamine usage on immediate graft function after kidney transplantation [3, 84, 85].

For long time dopamine has been considered as “first line” vasopressor and was frequently used at the intensive care unit (ICU) to improve organ perfusion. Acute renal failure (ARF) is common in critically ill patients and is associated with a high mortality rate. Because animal models use ischemia to induce experimental ARF, there is the widespread belief that lack of blood flow is responsible for ARF. Low-dose dopamine (LDD) has been shown to increase renal blood flow in animal and in human volunteers. Thus, it has been administered to humans for almost 3 decades in the belief that it would lead to renal arterial vasodilation and increase renal blood flow (RBF). However, 2 meta-analyses and a large double-blind, prospective, multiple-center, randomized controlled trial have failed to demonstrate that dopamine protects the kidney in critically ill patients with ARF [86, 87]. Currently, there is insufficient evidence to support the use of renal-dose dopamine in the intensive care unit. Nevertheless, it must be emphasized that the meta-analysis studies were unable to detect increasing adverse effects of LDD at the level of statistical significance.

Dopamine, an alpha and beta-sympathomimetic drug is an endogenous catecholamine that stimulates the release of adrenalin. It acts via binding to alpha and beta1 receptors as well as dopaminergic receptors.

![Chemical structure of dopamine](image)

**Fig. 2 Chemical structure of dopamine**

The benefit of donor catecholamine usage is controversially discussed in transplantation literature. Some studies have suggested the use of catecholamines at least in hemodynamic unstable donors [88, 89], while other studies showed that donor dopamine usages was associated with a poor transplantation outcome [90] or were not able to show any effect [91]. A positive influence of donor dopamine usage could be related to improved organ perfusion [92]. Chammoro et al suggested for heart transplantation that catecholamines should be used in donors without limitation to improve coronary blood circulation and because of possible
immunomodulatory effects [93]. It is generally believed that activation of the immune system depends on the presence of so called danger signals [94]. These signals activate the innate immune system which in turn leads to inflammation. In the absence of alloantigens the inflammatory response is self-limiting, but in the presence of foreign antigens it leads to acquired immunity, perpetuation of the inflammatory response and tissue destruction [94]. As suggested from studies in brain dead animal models, it is most likely that brain death results in activation of the innate immune system [58, 95, 96]. Also ischemia and subsequent reperfusion are conditions in which danger signals may lead to tissue inflammation [41-43]. Ischemia results in an increased expression of MHC class II molecules in the kidney [97, 98] and in necrosis and apoptosis of renal tubular cells [36] [35, 36, 99, 100]. Because we could show that dopamine treatment of brain dead donors have a beneficial effect on delayed-graft function and long-term renal allograft survival [3, 84], it is conceivable to think that donor dopamine treatment might reduce danger signals.

Indeed, dopamine is able to induce the expression of the protective gene heme oxygenase 1 (HO-1) in endothelial cells [101], to delay the expression of adhesion molecules after TNFα stimulation, to inhibit the production of chemokines in renal tubular epithelial and endothelial cells [102] and to protect endothelial cells against cold preservation injury [103, 104]. In an animal model we could observe that pre-treatment of donor rats with dopamine improves early graft function, protects endothelial cells against cold preservation injury and improves renal function after warm ischemia [105, 106]. Moreover, treatment of brain-dead rats with dopamine reduces monocyte infiltration and improves renal perfusion in donor kidneys [107].

**Heme oxygenase-1**

In animal models it has already been shown that an up-regulation of the antioxidative enzyme hemoxygenase-1 (HO-1) in the donor improves long-term graft survival [108-110]. HO-1 exerts anti-inflammatory effects and seems to be protective in transplantation medicine [108-110].

The HO system comprises several iso-enzymes [111, 112], of which the inducible HO-1 iso-enzyme seems to be particularly important as anti-inflammatory mediator [113-116]. Heme oxygenases are the rate-limiting enzymes in degradation of heme into carbon monoxide (CO), Fe^{2+} and biliverdin, the latter being subsequently converted to bilirubin [117, 118]. In addition to its unambiguous role in oxidant induced injury [119-121], unequivocal evidence demonstrates that the heme oxygenase system (HO) also takes part in control of inflammatory processes [113-115, 122]. A possible mediator by which HO-1 exerts its beneficial effect is
carbon monoxide (CO). As CO itself up-regulates HO-1 expression, perpetuation of the anti-inflammatory effect could be expected [123].

Otterbein et al and Sethi et al clearly demonstrated the anti-inflammatory potential of CO in macrophages and monocytes [113] as well as in endothelial cells [124, 125]. The salutary effect of CO has also been shown for organ transplantation and ischemia reperfusion injury in animal models [126, 127]. Recently a new class of molecules, termed CO-releasing molecules (CORM), has been described that are composed of transition metals carbonyls and have the ability to liberate CO under appropriate conditions [128]. In particular, CORM-3 (tricarbonylchloro(glyconato)ruthenium(II)) and CORM-A1 (sodium boranocarbonate), both of which are fully water soluble, rapidly liberate CO when dissolved in physiological solutions. These molecules might therefore be of therapeutically interest to modulate ongoing inflammatory reactions by delivering CO in a controllable fashion [129].
Aims of the study

Brain death and cold preservation are both alloantigen-independent factors that are involved in pre-transplantation injury and consequently negatively affects transplantation outcome [32-34]. The aim of this studies was to investigate how these factors influence graft quality and how appropriate intervention in the donor, or in the allograft during cold preservation, can influence organ function after transplantation.

For this purpose it is important to investigate the progressive changes during brain death in more detail. Previous studies could already show a correlation between brain injury and autonomic neurologic dysfunction measured by heart rate variability in humans [81, 82]. There is however no evidence available demonstrating that impairment of the parasympathetic nervous system occurs in the course of brain death and that this might contribute to inflammation in end-organs. This has been studied in chapter 2.

Static cold storage is the most commonly used preservation method in organ transplantation. Yet prolonged cold preservation is associated with a poor transplantation outcome. In chapter 3 we addressed if cold preservation and brain death are synergistic with respect to pre- and post-transplantation injury.

To reduce pre-transplantation injury, new strategies in donor management and/or organ procurement are warranted. In two retrospective clinical studies our group could already show that catecholamine donor pre-treatment results in a lower incidence of acute rejection episodes and a substantially better graft survival [3, 84, 85]. Although the precise mechanisms by which DA may improve the transplantation outcome after renal transplantation are not entirely known thus far, several mechanisms have been suggested. In chapter 4 we tested the hypothesis that the anti-inflammatory effect of DA is independent of blood pressure stabilization. In a subsequent study that we conducted, we addressed if dopamine treatment of brain-dead donor rats can influence early renal function and renal inflammation after transplantation. The results of this study are described in chapter 5. Since in the latter study cold preservation was not applied, we addressed in chapter 6 the question if donor dopamine treatment is protective when grafts are subjected to 24 hrs of cold preservation.

Because the anti-inflammatory potential and cytoprotective properties of CO already have been shown [113, 124, 125], we tested in chapter 7 if addition of CO-releasing molecules to the preservation solution can prevent endothelial damage and thereby maintaining vascular function. Since it is generally believed that vascular damage can give rise to intima hyperplasia, we also tested if the protective effect of CO-releasing molecules was translated into reduction of intima hyperplasia, in aortic grafts harvested 2 months after transplantation.
Chapter 1 – General Introduction and Aims of the Study

References

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