The alloantigen-independent factors brain death and cold ischemia
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2009

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Höger, S. (2009). The alloantigen-independent factors brain death and cold ischemia: prospects for a better graft survival through different donor management concepts Simone Höger

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Chapter 8 – General Discussion

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Summary

There is general consensus that amongst the renal replacement modalities, renal transplantation is the most desirable therapy for patients with end-stage renal failure, as it not only significantly improves quality of life but also reduces mortality risk compared to patients on dialysis [1]. Yet, the increasing number of patients on the waiting list and the relative stable supply of donors will continue to enlarge the negative balance between organ donors and recipients in the coming years. Therefore more and more donors that do not fulfil the optimal criteria for organ donation are now being used to increase the potential donor pool. The caveat however is, that with the use of renal allografts from marginal donors there is in general a higher frequency for delayed graft function observed, which might translate in a poorer long-term graft survival [2]. Because marginal donors to a large extent will continue to contribute to the donor pool, new concepts are warranted to limit organ damage before transplantation and to maintain organ quality.

Brain death - and cold storage associated injury are the leading causes for pre-transplant deterioration of organ quality. Inasmuch as brain death can lead to hemodynamic instability [3] and promotes a proinflammatory state of the graft [4-7], optimal donor management is of critical importance to the success of transplantation.

Static cold storage is routinely used to make organ allocation possible. Extended cold ischemia time however affects graft function and graft survival negatively [8-10]. Also static cold storage, which was developed in the area of optimal donors, might not be ideal anymore when facing a qualitative change of deceased donors.

The studies presented in this thesis were aimed to understand how brain death and cold storage, either alone or in combination, influence organ quality or function after transplantation. Moreover, attempts were made to reduce pre-transplantation injury through donor or organ pre-conditioning.

Although there is clear evidence that brain death induces an inflammatory response in a number of organs [4-6, 11, 12], little is known about the mechanism by which this is mediated. Because vagus nerve activity ceases after brain injury [13, 14], impairment of the cholinergic
inflammatory reflex might be a possible explanation for the inflammatory response in end-organ. Using an animal model we tested in Chapter 2 the hypothesis that brain death induced inflammation correlates with autonomic neurological dysfunction and that vagus nerve stimulation could attenuate the pro-inflammatory state of organs in brain-dead donors.

We could demonstrate that heart rate variability (HRV) decreased in brain-dead animals, suggesting a decreased vagal tone [15, 16]. In affymetrix chip analysis we could confirm with Koodstaal et al. [17, 18] that a number of pro-inflammatory genes are upregulated in the small intestine of brain-dead animals. Beyond it we could show that stimulation of the vagus nerve could normalize the expression of these genes. Also a number of pro-inflammatory genes, e.g. chemokines, adhesion molecules and signalling molecules, which were not influenced by brain death, were modulated by vagus nerve stimulation. Systemically we observed a significant increase of serum TNFα during the course of brain death, but not in brain-dead animals receiving vagus nerve stimulation. Using qPCR we could show that some of the changes in gene expression during brain death and intervention were also observed in other organs such as heart, kidney and liver. Most interestingly, renal function was clearly better when allografts obtained from brain-dead animals receiving vagus nerve stimulation were transplanted in syngeneic recipients, compared to allografts from brain-dead animals that did not receive vagus nerve stimulation. Hence we conclude in Chapter 2 that impairment of the cholinergic inflammatory reflex, at least partly, might underlie brain death induced inflammation.

Although static cold storage is the most widely used modality for preserving organs from deceased donors, it is also a major cause for pre-transplantation injury of grafts [9, 19-21]. In Chapter 3 we addressed to what extent brain death and cold ischemia have a synergistic effect on organ damage and tissue inflammation after renal transplantation in rats. We could show that cold preservation increases DNA damage in renal tissue before implantation. This is in line with other groups showing that prolonged cold ischemia increases apoptosis and necrosis in renal tubular cells [22-24]. Salahudeen also reported that prolonged preservation of cadaveric kidneys is a significant predictor for graft loss [19, 22]. Our study indicates that brain death augments DNA damage when grafts are subsequently subjected to prolonged cold preservation. Brain death can lead to hemodynamic instability and therefore to hypoperfusion in the kidney. This may in turn make the renal tissue more prone to cold ischemic damage. Ten days after transplantation renal inflammation was significantly higher in grafts that were subjected to cold preservation.
Brain death aggravated renal inflammation; particularly vasculitis was significantly more severe in transplanted grafts from brain-dead animals that were exposed to cold preservation compared to cold preserved grafts from the living donors. Sanchez-Fructuoso et al [25] also found that brain death is a risk factor for acute vascular rejection, but the role of cold ischemia was not assessed in their study. The findings of Simpkins et al [26] suggest that cold ischemia time is not a risk factor for acute interstitial rejection when grafts are obtained from living donors. This is not surprising because cold ischemia time of grafts from living donors is in general short and transplantation outcome is much better compared to organs retrieved from deceased donors. Because cold ischemia time didn't exceed 8 hours in the study of Simpkins, while in our study a cold ischemia time of study 24 hours was applied, this may explain the different findings between both studies. Brain death was associated with papillary necrosis in transplanted grafts, which might be related to hypoperfusion of the renal tissue during the state of brain death. Subsequently ischemic damage could occur, resulting in papillary necrosis [27, 28]. With exception of VEGF and IL-10 no significant differences in mRNA expression for cytokines or growth factors in renal tissue were found between the groups. VEGF was strongly up-regulated in grafts from brain-dead animals compared to grafts from living donors. Since VEGF is up-regulated by hypoxia in a variety of organs [29-31], the increased expression might be related to tissue ischemia. Ten days after transplantation IL10 expression was still increased in grafts from brain-dead donor rats, probably as a response to renal inflammation. In conclusion we demonstrate in Chapter 3 that brain death amplifies tissue damage before and after transplantation when renal allografts are subjected to prolonged cold ischemia. This once more stresses the importance of minimizing cold ischemia time in grafts from deceased donors in order to prevent aggravation of graft injury and hence to improve transplantation outcome.

Patients with irreversible brain injury are frequently treated on the intensive care unit with catecholamines to stabilize blood pressure [32-35]. Interestingly, the use of catecholamines on potential donors significantly influences transplantation outcome [36, 37]. In brain death models dopamine application not only improves mean arterial pressure, but also reduces mononuclear cell infiltration in the kidney [38]. In Chapter 4 we tested the hypothesis that the anti-inflammatory effect of dopamine is independent of blood pressure stabilisation. We investigated the involvement of α- and β-adrenergic- as well as dopaminergic receptors in this regard.
As mentioned above, hypotension during brain death may subsequently lead to hypoperfusion of organs, tissue ischemia and consequently to generation of reactive oxygen species [12, 39]. We have recently demonstrated in an animal model, that dopamine treatment during brain death improves renal perfusion [38]. A correlation between hemodynamic stabilisation and reduced renal inflammation has also been demonstrated by other groups [11, 12, 40, 41]. Therefore blood pressure stabilization to physiological values seems to be auspicious in preventing tissue injury in BD donors before organ procurement. Dopamine applied in a clinically relevant dosage also exerts beneficial effects on renal inflammation independently from hemodynamic stabilisation.

We could show that 24 hours of dopamine pre-treatment (stopping directly before brain death induction) reduced monocyte infiltration, although mean arterial pressure was not influenced during brain death. Moreover D-receptor antagonists abrogated the anti-inflammatory effect of dopamine without changing its blood pressure stabilising properties, whereas application of a D-receptor agonist reduced monocyte infiltration, but it did not improve mean arterial pressure during brain death. Hence dopamine seems to have a direct anti-inflammatory effect mediated via D-receptor stimulation.

In BD animals renal HO-1 was significantly upregulated. Dopamine and the D-receptor agonist pergolide abrogated the upregulation of HO-1. The findings on HO-1 seems to be in conflict with previous in vitro data reported by our group, showing that HO-1 expression was upregulated by dopamine [42]. Since HO-1 expression is upregulated during cellular stress, e.g. ischemia, it is conceivable that up-regulation of HO-1 during brain death was related to cellular stress through hemodynamic disturbances, hypothermia and coagulopathy [3]. If dopamine reduces cellular stress than up-regulation of HO-1 will accordingly not occur. It also must be emphasized that up-regulation of HO-1 in vitro only occurred at relative high dopamine concentration and was clearly dependent on the pro-oxidative properties of dopamine [42]. In vivo these concentrations were not reached. In conclusion, we demonstrate in Chapter 4 that hemodynamic stabilisation is beneficial to limit BD induced inflammation. Nevertheless, the anti-inflammatory effect of dopamine involves more than blood pressure stabilisation. D-receptor stimulation and even non-receptor mediated effects of dopamine might collectively contribute to the anti-inflammatory effect of dopamine in brain dead donors.

Since dopamine treatment reduces the inflammatory response in brain-dead donor animals [38, 43], we addressed in Chapter 5 if dopamine treatment of brain-dead donor rats can influence
early renal function and renal inflammation after transplantation. A main finding of this study is that donor dopamine treatment improved renal function in the recipients independently of changes of renal function in the donors. In the human transplantation situation, early renal function has a beneficial effect on transplantation outcome [8, 44, 45] and predicts 5 years graft survival [46]. Improvement in early renal function by donor dopamine treatment might therefore significantly improve long term graft prognosis [36, 37]. Ten days after transplantation the number of graft infiltrating cells was significantly reduced in the donor dopamine treated group. This was reflected by lower Banff 97 tubulitis and interstitial inflammation score. Because tubulitis is a hallmark for acute interstitial rejection after renal transplantation in men, our data indicate that donor dopamine treatment may influence the process leading to acute interstitial rejection. Our findings suggest that dopamine treatment reduces monocyte infiltration during brain death and hence reduces the number of passenger leukocyte in the graft [38, 43]. These mobile cells migrate out of the graft into secondary lymphoid organs where they can initiate an immune response against the graft [47]. A significant reduction in CINC-1 expression, a rat homologue for IL-8, might also contribute to a decreased inflammatory response in the transplanted renal allograft. Because dopamine also ameliorates ischemia/reperfusion injury [48] this could contribute to a reduced inflammation and an improvement of renal function. In conclusion we demonstrate in Chapter 5 that donor dopamine treatment during brain death may provide a benefit on graft survival both by improving early renal function after transplantation and by reducing renal inflammation.

Donor dopamine treatment has a beneficial effect on transplantation outcome in the Fisher to Lewis model. In this model however, donor and recipient only differ in minor histocompatibility antigens [49], while in men renal transplantation is in the majority of cases performed across major histocompatibility complex barriers. In Chapter 6 we used the MHC discordant Brown Norway to Lewis transplantation model, a model in which acute rejection is known to occur [50], to address if donor dopamine treatment is also effective in this combination, even when allografts are subjected to prolonged cold preservation. One major finding of this study was that donor dopamine treatment significantly reduced the severity of acute rejection. This was indicated by lower Banff tubulitis scores.

Cold preservation and ischemia reperfusion injury are known to be associated with inflammation [51-54]. We could show that donor dopamine pre-treatment of Brown Norway donors reduces LTα, TNFα, IL-1β and IL-2, mRNA expression in the renal allografts after transplantation. These
cytokines might modulate inflammation stimulating monocytes, T-cell proliferation and by increasing the expression of adhesion molecules on the endothelium [55-57]. In our study donor dopamine treatment significantly reduced the number of infiltrating MHC class II + and CD3 + cells, analyzed 5 and 10 days after transplantation. A reduction of IL-2 expression in dopamine treated grafts might be due to the reduced number of infiltrated CD3 + cells in the transplanted kidneys. Acute rejection is mediated by T-cells that recognize and destroy tubular cells in an allo-antigen dependent fashion [58-62]. The severity of tubulitis was significantly decreased in dopamine treated allografts. This is in accordance with the clinical studies of Schnuelle et al [37] who found that donor dopamine usage was associated with less acute rejection episodes. Hence we demonstrate in Chapter 6 that donor dopamine treatment improves transplantation outcome even when transplantation is performed in MHC discordant donor recipient combinations.

With regard to protection against cold storage induced damage, we also investigated a compound belonging to the class of CO-Releasing Molecules (CORM). Apart from its anti-inflammatory effects [63], [64, 65], CO might protect the endothelium by virtue of its iron binding properties. Iron chelators, e.g. deferoxamine, are known to be protective against preservation injury [66]. Because cold preservation time is a risk factor for chronic allograft vasculopathy [67] it is of utmost importance to protect the endothelium during cold preservation. A characteristic hallmark for chronic allograft vasculopathy, a leading cause for chronic organ loss after transplantation [36, 68], is intima hyperplasia often leading to vascular obliteration. In Chapter 7, we investigated the beneficial effect of CORM-3 on cold preservation injury in HUVECs and its influence on vascular remodelling and vascular function in a syngeneic rat aorta transplantation model. We could show that CORM-3 protected endothelial cells against cold preservation injury via liberation of CO. Cold storage induced endothelial denudation and intercellular gap formation in isolated abdominal aortas of rats, while this was prevented when CORM-3 was added to the preservation solution. Vascular function was significantly impaired after 24 hrs of cold ischemia. This was mediated by impairment of endothelial NO production, since vessel relaxation by addition of the NO donor SNP was not affected after cold ischemia. Two months after aorta transplantation, intima hyperplasia was detected in all grafts, which were subjected to 24 hrs of cold preservation. Addition of CORM-3 to UW-solution significantly inhibited intima hyperplasia. In conclusion, we demonstrate in Chapter 7 that endothelial damage during cold preservation is an eligible
condition for intima hyperplasia after transplantation even in the absence of an anti-donor immune response. Addition of CORM-3 to UW-solution prevents endothelial damage, maintains vascular function and limits intima hyperplasia. Further studies are nevertheless warranted to assess the efficacy of this approach for transplantation outcome in vascularized allogeneic solid organs.
Conclusions and Future Perspectives

As it is generally acknowledged that kidney transplants from poorly matched unrelated living donors show a better graft survival compared to that from well matched deceased donors [69], the concept of reducing pre-transplantation tissue injury as a mean to improve long-term allograft survival has received more appreciation in recent years. Both donor brain death and cold preservation are major causes of pre-transplantation injury, and hence represent genuine targets for the development of new strategies in donor management and organ preservation.

The studies presented in this thesis not only give more insights on the impact of allo-antigen independent factors, i.e. brain death and cold preservation, on transplantation outcome, but also show how their negative influence can effectively be diminished by donor pre-conditioning and / or changes in cold preservation. The main lessons that should be learned from these studies are the following. First, brain death induced inflammation might be linked to impairment of the cholinergic inflammatory reflex, a finding that has previously not been recognized. Second, donor dopamine pre-conditioning is very effective to improve transplantation outcome. It reduces inflammation during brain death, possibly by improving hemodynamics but also because of its direct anti-inflammatory effect. Moreover, dopamine pre-treatment seems to protect the allograft against cold preservation injury. This is also found in a recently finished prospective randomized clinical study on donor dopamine usage, demonstrating that the salutary effect of dopamine on delayed graft function is more pronounced when cold ischemia time is long (Schnülle et al, unpublished data). Third, brain death and cold preservation are synergistic, i.e. they aggravate tissue damage before and after transplantation. Fourth, protection of the endothelium during cold preservation might reduce chronic allograft vasculopathy. This could be achieved by implementation of CO in preservation solutions.

The main question that I would like to address now is what are the clinical implications of our findings and what are the future perspectives for transplantation research. Impairment of the vagus nerve has been described in brain-injured patients [13, 14], it is therefore conceivable that vagus nerve stimulation during brain death would have a beneficial effect on the inflammatory response in end-organs. Although it is tempting to suggest that vagus nerve stimulation should be performed in deceased donors, our data are still preliminary and should be confirmed in allo-transplantation models. Yet, vagus nerve stimulation already has shown to be
an effective activator of the anti-inflammatory pathway in several in vivo models [70-72]. The issue of timing and duration of vagus nerve stimulation in the brain death model also needs to be addressed.

Our study once more stresses the importance of minimizing cold ischemia time in grafts from deceased donors. However, for logistical reasons this can not always be achieved. New strategies in donor management and/or organ procurement aimed to minimize pre-transplant injury are therefore warranted. Promising prospects might be donor pre-conditioning [36, 37, 68] and hypothermic machine perfusion [73, 74]

One of the beneficial effects of donor dopamine usage is clearly related to protection against cold preservation injury. This effect is however not receptor mediated and independent of dopamine’s blood pressure stabilising properties. Recently we have developed a new dopamine derivative, i.e. n-octanoyl-dopamine (NOD) that is devoid of hemodynamic action and shows \textit{in vitro} a 40 x higher efficacy in its protective effect. Due to the fatty acid attached to the amine side chain, NOD is highly hydrophobic, making its clinical application difficult. This problem can be overcome by dissolving NOD in so called semi-fluorinated alkanes (SFA) and using emulsion hereof for intravenous application. The advantage of SFA emulsions is not only that they can be used as drug delivery system, but that they can also be used as oxygen carriers or carriers for carbon monoxide. Therefore SFA emulsions might represent a new technological platform, which has a potential use in donor management for systemic delivery of protective compounds to organ allografts. In addition, SFA emulsions might be used for other technologies of organ preservation, i.e. hypo- or normothermic machine perfusion, to deliver oxygen to the allograft.
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