Vagal stimulation in brain dead donor rats decreases chronic allograft nephropathy in recipients

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ABSTRACT

Background. Although it has been shown that a vagus nerve stimulation of brain dead (BD) donors leads to an improvement of renal function in recipients in an acute allograft rejection model, its influence on chronic allograft nephropathy is still unknown. In the present study, we assessed the influence of donor vagus nerve stimulation on survival, renal function and histology in a chronic allograft model.

Methods. Brain death was induced in Fisher rats, and electro-stimulation of the vagus nerve was applied in one group (BD + vagus) during the whole course of BD (6 h). Unstimulated BD Fisher donor rats served as controls. Allogeneic Lewis rats were used as recipients and no immunosuppressive medication was administered. Blood and urine samples were collected every second week. Banff classification was assessed from harvested allografts.

Results. Vagal stimulation of BD donors resulted in an improved survival of recipients. Long-term renal function was significantly better in these recipients as reflected by improved creatinine clearance. Banff classification revealed significantly reduced vasculopathy and less tubulopathy in the BD + vagus group.

Conclusions. In conclusion, our data demonstrate a long-lasting beneficial effect of vagus nerve stimulation in BD donors on the renal transplantation outcome. Hence, activation of the cholinergic anti-inflammatory pathway in BD donors may represent a novel therapeutic modality to reduce chronic allograft nephropathy without any side effects for the recipient.

Keywords: brain death, chronic allograft nephropathy, kidney transplantation, rat model, vagus nerve

INTRODUCTION

The vagus nerve innervates a variety of visceral organs and might be considered as the interface between the central nervous and the immune system. It is of eminent importance in the cholinergic anti-inflammatory pathway, also referred as inflammatory reflex [1]. The inflammatory reflex comprises sensory afferent and motor efferent branches that modulate cytokine responses. The motor neural arc of the reflex is called the anti-inflammatory pathway, and suppresses cytokine production via the release of the primary parasympathetic neurotransmitter acetylcholine, which binds to both nicotinic and muscarinic cholinergic receptors. The main nicotinic cholinergic receptor found on macrophages, i.e. the α7 subunit (α7nAChR) [2], is believed to be a pivotal functional component of the cholinergic anti-inflammatory pathway. While electro-stimulation of the vagus nerve down-regulates tumor necrosis factor-α serum levels in wild-type but not in α7 knockout mice, in vitro studies have unambiguously shown the efficacy of cholinergic agonists in models of sepsis, septic shock and ischaemia reperfusion injury (IRI) [4–6].

In line with the published human studies on brain injury and autonomic neurologic dysfunction [7–9], we have previously demonstrated that in brain dead (BD) donor rats, heart rate variability (HRV) is decreased, suggesting impairment of the parasympathetic nervous system. It is therefore conceivable that inappropriate activity of the cholinergic anti-inflammatory pathway might contribute to or exacerbate BD-induced inflammation in end organs. Indeed vagus nerve stimulation in BD rats results in down-regulation of pro-inflammatory genes in peripheral donor organs, but more importantly also improves early renal function in recipients receiving a renal allograft of such donors [10].

As the mismatch between supply and demand of deceased donor kidneys for transplantation continues to grow, the
Blood pressure. Each group consisted of a minimum of 11 control. All animals were treated with NaCl 0.9% to stabilize brain death induction. Untreated BD animals were used as a during the whole brain death period, starting 15 min before constant voltage stimulus (5 V, 2 ms, 1 Hz) was applied Farchant, Germany) on the cervical vagus nerve trunk. A pressure processor, FMI, Ober-Beerbach, Germany). In one group of animals, electrostimulation of the vagus nerve was performed (BD + vagus) by placing bipolar electrodes con- tinuously measured (6 h) in the donors by a femoral arterial pressure [mean arterial pressure (MAP), mm HG] was con- stantly monitored. Anesthesia was induced as formerly described [15]. Systemic blood pressure [mean arterial pressure (MAP), mm HG] was con- tinuously measured (6 h) in the donors by a femoral arterial catheter (Statham pressure transducer P23Db and a Gould pressure processor, FMI, Ober-Beerbach, Germany). In one group of animals, electrostimulation of the vagus nerve was performed (BD + vagus) by placing bipolar electrodes con- nected to a stimulation module (digi 3000, Promed GmbH, Farchant, Germany) on the cervical vagus nerve trunk. A constant voltage stimulus (5 V, 2 ms, 1 Hz) was applied during the whole brain death period, starting 15 min before brain death induction. Untreated BD animals were used as a control. All animals were treated with NaCl 0.9% to stabilize blood pressure. Each group consisted of a minimum of 11 animals.

Renal transplantation
To assess the influence of vagus nerve stimulation on the graft outcome, renal transplantsations were performed in the Fisher to Lewis (allogeneic) model. The left kidney was explanted 6 h after BD induction. During the cold storage time of 1 h, the kidneys were preserved in University of Wisconsin solution before they were implanted into unilateral nephrecto- mized recipients. In the present study, initially only a unilateral nephrectomy was performed in the recipients to reduce the number of dropouts due to complications caused by a decreased early renal function, since the beneficial influence of vagus nerve stimulation in BD donors on early renal function in allogeneic bilaterally nephrectomized recipients was already demonstrated in a previous study [10]. The second kidney of the recipient was removed 10 days after transplantation. Hence, baseline renal function was accordingly determined 2 weeks after transplantation. Immunosuppressive medication was not installed. Renal function was assessed in all recipients by the serum creatinine clearance calculated by using the following equation: clearance (mL min⁻¹ kg⁻¹) = [urinary Cr (mg/dL) × urinary volume (mL)/serum Cr (mg/dL)] × [1000/ body weight (g)] × [1/1440 (min)]. Therefore, animals were put in metabolic cages every second week after transplantation to collect 24-h urine. Both urine and blood parameters were assessed in parallel. Kidneys of the recipients were harvested 16 weeks after transplantation, unless the general condition of the animals required earlier termination.

Methods

Animals
Inbred male Fisher (F344, RT11) and Lewis (LEW, RT1) rats weighing 200–250 g were obtained from Charles River (Sulzfeld, Germany). Animals were kept under standard conditions and fed standard rodent chow and water ad libitum. All procedures were performed according to the Guide for the Care and Use of Laboratory Animals published by the National Academy of Sciences and were approved by the local authorities (RP Karlsruhe, AZ 35-9185.81/113/07).

Experimental protocol
Brain death and vagus nerve stimulation. Brain death was induced as formerly described [15–17]. Systemic blood pressure [mean arterial pressure (MAP), mm HG] was con- tinuously measured (6 h) in the donors by a femoral arterial catheter (Statham pressure transducer P23Db and a Gould pressure processor, FMI, Ober-Beerbach, Germany). In one group of animals, electrostimulation of the vagus nerve was performed (BD + vagus) by placing bipolar electrodes con- nected to a stimulation module (digi 3000, Promed GmbH, Farchant, Germany) on the cervical vagus nerve trunk. A constant voltage stimulus (5 V, 2 ms, 1 Hz) was applied during the whole brain death period, starting 15 min before brain death induction. Untreated BD animals were used as a control. All animals were treated with NaCl 0.9% to stabilize blood pressure. Each group consisted of a minimum of 11 animals.

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Histology
Paraffin embedding of harvested grafts was performed using routine procedures. Paraffin sections (4 µm) were fixed in 10% neutral buffered formalin. Histologic grading was per- formed according to the Banff 97 classification [18]. Histologic evaluation and grading included interstitial inflammation, tu- bulitis and intimal arteritis. The histologic grading scale was from 0 to 3 (0 = not present, 1 = mild alteration, 2 = moderate alteration and 3 = severe alteration) according to the Banff 97 criteria. Chronic allograft nephropathy was evaluated by scoring chronic glomerular changes, interstitial fibrosis, tubular atrophy and vascular intimal sclerosis. The grading scale was from 0 to 3 (0 = not present, 1 = mild alteration, 2 = moderate alteration and 3 = severe alteration).

Immunohistochemistry
Serial paraffin sections (4 µm) were fixed in 10% neutral buffered formalin for immunohistochemical staining. Immuno- histochemistry for ED1-positive monocytes was performed as described [19, 20].

Statistical analysis
For survival analysis, Kaplan-Meier curve and multivariate log-rank test were applied. For renal function and proteinuria ANOVA analysis of variance was applied. Statistical analysis for immunohistochemistry was performed using the Kruskal-Wallis test with an option for multiple comparisons. For analysis of light microscopy, Fisher’s exact test was applied (StatsDirect 2.2.8, Aswell, UK). A P-value of <0.05 was con- sidered significant.
RESULTS

Blood pressure monitoring in BD donors

Brain death induced profound haemodynamic alterations in both BD and BD + vagus groups. This was reflected by an initial increase in the mean arterial blood pressure (MAP), followed by a sharp decline and finally a stabilization of the MAP around 80 mmHg during the whole 6 h of BD (Figure 1). Vagus stimulation in BD rats does not affect the systemic blood pressure as already demonstrated in our previous study [10].

Animal survival

Renal allografts were transplanted in Lewis recipients unless the MAP in donor Fisher rats dropped <70 mmHg. This did not occur frequently (<10%) and was similar in both groups. Animal survival was significantly better in the BD + vagus group (P < 0.05). While in the BD control group (n = 12) only 1 animal survived 16 weeks, in the BD + vagus Group 5 out of 11 recipients survived 16 weeks (Figure 2).

Allograft function

In addition to improved animal survival, renal function was significantly better in the BD + vagus when compared with the BD group. Serum creatinine and serum urea were clearly lower in recipients who received a renal allograft from a BD + vagus donor (BD versus BD + vagus s-crea, P < 0.05; s-urea, P < 0.05) (Figures 3 and 4). Furthermore, the creatinine clearance revealed a significantly better transplant function in the BD + vagus group during the 16-week observation period (P < 0.01) (Figure 5). Proteinuria was similar in both the groups until Week 10. Although in Week 12, proteinuria tended to increase more in the BD group and even stronger at Week 14, this did not reach statistical significance (Figure 6).

Light microscopy

The light microscopy examinations were performed by a blinded pathologist. If recipients were in a bad general condition with severe distress and were expected to die within the next day, they were prematurely sacrificed according to the legislation on animal welfare. Light microscopy was performed on grafts obtained from prematurely sacrificed rats and on grafts obtained from rats that survived the whole observation period (16 weeks) (Figure 7). Banff classification of the transplanted renal grafts revealed a significant lower intimal arteritis (P < 0.01) and tubulitis (P < 0.05) score in the BD + vagus group (Table 1). There was no difference in interstitial inflammation. Tubular atrophy was significantly more pronounced in the control group (BD versus BD + vagus, P < 0.05), whereas no significant difference in vascular sclerosis and interstitial fibrosis (Table 2) was observed between the groups.

Immunohistochemistry

We also assessed monocyte/macrophage infiltration by immunohistochemistry in both the groups. In line with the Banff classification, interstitial inflammatory scores, there was no statistical difference in the number of ED1-positive cells,
although there was a tendency that in renal grafts from BD + vagus the number of ED1-positive cells was slightly reduced (BD + vagus versus BD: 32 ± 1 versus 37 ± 10, P = NS) (data not shown).

**DISCUSSION**

The present study was conducted to assess the effects of vagus nerve stimulation in BD donors on the long-term transplantation outcome in the Fisher to Lewis model of chronic renal allograft nephropathy.

The main findings are the following. First, vagal stimulation of BD donors improved survival of the recipients. Second, vagus nerve stimulation of BD donors improved renal function in the recipients. This was reflected by a better serum creatinine, better serum urea and better creatinine clearance. Third, vagus nerve stimulation of BD donors significantly reduced intimal arteritis, tubulitis and chronic tubulopathy in the grafts.

It is generally accepted that brain death induces inflammation in end organs, enhances subsequent ischaemia-reperfusion injury and accelerates acute and chronic rejection in transplanted grafts [19–25]. Consequently, several strategies have been developed to limit brain death-induced inflammation and data from preclinical [15, 16, 26] and clinical studies [14, 27–30] show promising results on early graft function.

Although it is still not completely clarified how BD evokes inflammation in end organs, we have evidence that an impairment of the vagus nerve might at least partly be involved in this process. It was shown that there is a correlation between brain injury and the HRV which implements an impairment of the vagus nerve [7, 31–33]. Furthermore we have shown that vagus nerve activity decreases in the course of BD, indicated by a decreasing HRV and that stimulation of vagal activity in BD donors has a considerable anti-inflammatory potential in end organs [10]. Vagus nerve stimulation leads to a better early renal function in recipients, most likely as a consequence of improved organ allograft quality, i.e. less inflammation.

The results of the chronic allogenic model indicate a long-lasting protective effect on the graft after vagus nerve stimulation in Fisher donor rats. To reduce confounding factors to a minimum, we did not administer any immunosuppressive medication. Both functional and histological parameters were significantly better in the BD + vagus group which is reflected in a significantly better survival rate in the BD group. Due to ethical and legal reasons, most animals had to be sacrificed before the primary end point due to their bad clinical condition (only 1/12 in the BD group and only 5/12 in the BD + vagus group survived 16 weeks). The decision for premature terminating recipients was made according to the objective criteria which are given by the regional council. Nonetheless, all renal allografts were analysed histologically, and revealed less intimal arteritis, tubulitis and tubular atrophy in the BD + vagus group while interstitial inflammation and fibrosis did not differ significantly from the BD group. One possible explanation why we were not able to demonstrate a reduced interstitial inflammation based on the Banff classification system might be due to the definition of the highest class of interstitial inflammation (i3), which is...
defined as an inflammation of >50% of the parenchyma. Thus, although all biopsies of both groups were equally classified as i3, it does not rule out a significant difference of interstitial inflammation between the two groups. Nevertheless, we decided to include Banff classification in this study as it is the standard classification system for renal allograft pathology. Consistent with histological findings, renal function was significantly improved in BD + vagus recipients. Progressive renal allograft dysfunction resulting from cumulative histologic damage to the allograft is the major cause of late renal allograft loss. In particular, acute vascular rejection remains to be a clinical problem and an important cause for early and late graft loss [34, 35]. Although our data indicate that intimal arteritis is significantly reduced in the BD + vagus group, the more chronic vascular changes, i.e. intimal sclerosis, were not significantly different, which might be caused by the significant earlier death of a majority of the animals in the untreated group.

Table 1. Histological Banff classification of renal grafts after transplantation

<table>
<thead>
<tr>
<th>Banff (%)</th>
<th>BD (n = 11)</th>
<th>BD + vagus (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>i0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>i1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>i2</td>
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</tr>
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<td>100</td>
</tr>
<tr>
<td>t0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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</tr>
<tr>
<td>t2</td>
<td>50</td>
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<td>100</td>
</tr>
<tr>
<td>v2</td>
<td>58</td>
<td>0</td>
</tr>
<tr>
<td>v3</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Severity of interstitial inflammation (i), tubulitis (t) and intimal arteritis (v) is indicated by the following grading scale: 0, not present; 1, mild alteration; 2, moderate alteration and 3, severe alteration. Severity is presented in percentage of animals with a given score.

Table 2. Histological scoring of chronic nephropathy

<table>
<thead>
<tr>
<th>Chronic nephropathy (%)</th>
<th>BD (n = 12)</th>
<th>BD + vagus (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic glomerular change</td>
<td>cg0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>cg1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>cg2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>cg3</td>
<td>100</td>
</tr>
<tr>
<td>Interstitial fibrosis</td>
<td>ci0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>ci1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>ci2</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>ci3</td>
<td>92</td>
</tr>
<tr>
<td>Tubular atrophy</td>
<td>ct0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>ct1</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>ct2</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>ct3</td>
<td>67</td>
</tr>
<tr>
<td>Vascular intimal sclerosis</td>
<td>cv0</td>
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</tr>
<tr>
<td></td>
<td>cv1</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>cv2</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>cv3</td>
<td>0</td>
</tr>
</tbody>
</table>

Severity of glomerular changes, interstitial fibrosis, tubular atrophy and vascular intimal sclerosis is indicated by the following grading scale: 0, not present; 1, mild alteration; 2, moderate alteration and 3, severe alteration. Severity is presented in percentage of animals with a given score. *P < 0.05.

In line with our previous study, the current data suggest that stimulation of the anti-inflammatory pathway in BD donors by vagus nerve stimulation might be an effective way to improve transplantation outcome in renal allograft recipients. Whether this underlies the anti-inflammatory properties of the cholinergic inflammatory reflex in the donor or whether this is mediated by the protective effects of vagus stimulation on IRI per se is still elusive. Irrespective of its mode of action, our study demonstrates a long-lasting protective effect of vagal stimulation in BD donors in a model of chronic allograft
nephropathy. Hence, it may be a new promising treatment modality in BD donors to improve organ quality and to reduce chronic allograft nephropathy without any side effects for the recipient.

ACKNOWLEDGEMENTS

We would like to acknowledge Susanne Behr and Katharina Prem for their excellent technical support. S. H. and J. F. contributed equally to this work.

CONFLICT OF INTEREST STATEMENT

None declared.

REFERENCES


Received for publication: 20.6.2013; Accepted in revised form: 17.9.2013