Hypertension increases expression of growth factors and MHC II in chronic allograft nephropathy

RALF SCHINDLER, STEFAN G. TULLIUS, YAKUP TANRIVER, KERSTIN NOACK, YE QUN, JAN-STEFFEN JÜRGENSEN, and ULRICH FREI

Department of Nephrology and Internal Intensive Care Medicine and Department of Surgery, Universitätsklinikum Charité, Campus Virchow Klinikum, Humboldt University, Berlin, Germany

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Background. Hypertension of the recipient is strongly associated with chronic allograft nephropathy. It is unclear, however, whether hypertension is the cause or the consequence of chronic allograft nephropathy.

Methods. The present study was performed in the Fisher to Lewis rat kidney transplant model. Transplanted rats (N = eight in each group) received either no treatment or were made hypertensive by administration of deoxycorticosteron acetate (DOCA) and salt. Proteinuria and systolic blood pressure was measured monthly, grafts were harvested at 3 and 6 months for semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) and for immunohistology.

Results. Systolic blood pressure was markedly elevated in rats receiving DOCA/salt. Allografts of hypertensive animals contained significantly more cells expressing the proliferating cell nuclear antigen compared to isografts and to allografts from normotensive animals (P < 0.05). Histologic staining and mRNA expression of major histocompatibility complex (MHC) II was markedly increased in allografts of hypertensive animals compared to all other groups (P < 0.05). Expression of mRNA for platelet-derived growth factor-B (PDGF-B), transforming growth factor-β (TGF-β) and collagen was higher in allografts than in isografts and was highest in hypertensive animals.

Conclusion. We conclude that hypertension augments the expression of growth factors in the allograft possibly aggravating the intimal hyperplasia observed in chronic allograft nephropathy. By increasing the expression of MHC II antigens, hypertension may render the allograft more susceptible to alloantigen-dependent damage. Hypertension and alloantigen-dependent factors appear to exert additive or synergistic effects on inflammatory pathways leading to graft injury.

An ill-defined entity called chronic rejection or chronic allograft nephropathy remains the leading cause of graft loss after the first year of transplantation, particularly if death with a functioning graft is excluded as a cause of late graft failure [1]. Traditionally, the causes of chronic allograft nephropathy have been divided into alloantigen-dependent (immunologic) and alloantigen-independent (nonimmunologic) factors. The former include major histocompatibility complex (MHC)-mismatching and acute rejection episodes [2], the latter including hyperfiltration [3], conditions of the brain-death donor [4], and hypertension of the recipient. For many years, it has been known that hypertension is associated with graft failure. Cheigh et al [5] reported that graft survival was significantly inferior in hypertensive patients. Modena et al [6] observed an association between systolic and diastolic blood pressure and the pace of deterioration of graft function. Opelz, Wujciak, and Ritz [7] reported a highly significant correlation between posttransplant blood pressure and long-term graft outcome in more than 29,000 patients. Reports by Mange et al [8] confirmed these observations. In this study, the relative risk for graft failure per 10 mm Hg increase in mean arterial blood pressure was 1.30 [8]. An association between hypertension and deterioration of renal function does not prove a causal relationship since hypertension after transplantation might simply be the result of worsening graft function rather than vice versa. Retrospective studies cannot differentiate between cause and effect. The first evidence that hypertension per se may lead to graft damage was the observation that not only hypertension after transplantation, but also hypertension before transplantation is associated with later chronic allograft nephropathy [9].

Hypertension induces inflammatory changes in native kidneys, such as expression of intercellular adhesion molecules (ICAM-1) and interstitial leukocyte infiltration [10]. In the hypertensive rat remnant kidney model (an alloantigen-independent model of injury), expression of growth factors like platelet-derived growth factor-B

Key words: hypertension, intimal hyperplasia, chronic allograft nephropathy, DOCA/salt.

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(PDGF-B) is observed before glomerulosclerosis develops [11]. Moreover, the immunosuppressive drug myco-phenolate mofetil is able to attenuate renal injury in this model [12], indicating that hypertension after renal ablation causes renal injury through immune mecha- 
isms. On the other hand, superimposed renovascular hypertension aggravates renal damage in immunologic-mediated renal diseases such as antiglomerular basement membrane (GBM) nephritis [13] indicating that hyper- 
tension acts synergistically with immunologic factors on renal injury. To study the mechanisms by which hyper- 
tension may contribute to chronic allograft nephropathy, we investigated the effect of hypertension in a model of 
chronic rejection on renal pathology and expression of growth factors known to contribute to renal damage.

METHODS

Renal transplantation

Experiments were performed with male Fisher (F344) and Lewis rats (Harlan-Winkelmann, Borchen, Ger-
many). Rats were housed in standard plastic cages and maintained in a temperature- and humidity-controlled 
environment. The animals had access to either standard chow food or chow food containing 5% NaCl and tap 
water ad libitum. The local governmental committee for animal welfare approved the experiments.

Renal transplantation was performed as described pre-
viously [14]. F344 rats served as donors and Lewis rats as recipients. Under ether anesthesia, the left kidney was 
isolated, excised, and perfused with cold University of Washington (UW) solution. The kidney was transplanted 
orthopotically into a Lewis rat whose left kidney had been removed. Renal artery, vein, and ureter were anas-
tomosed end to end using 10-0 Prolene sutures. The Approximately 100 mg of renal tissue was homoge-
nized in 4 mol/L guanidine isothiocyanate (GITC) (Sigma 
Chemical Co., Deisenhofen, Germany). Four animals per 
group were sacrificed at 3 months after transplantation, 
the remaining four animals at 6 months after transplanta-
tion. At sacrifice, the aorta was cannulated with a 16 
gauge needle under anesthesia with ether and ketamine 
(100 mg/kg, administered intraperitoneally). The kidney 
was flushed with 50 mL phosphate-buffered saline (PBS) 
and fixed in situ with 50 mL 3% paraformaldehyde in 
PBS. The kidney was divided in three parts. One third 
was snap-frozen for RNA isolation, one third was em-
bodied in paraffin, and one third was immersed in 3% 
paraformaldehyde/100 mmol sucrose/100 mmol cacodyl-
ate overnight and then snap-frozen for immunohisto-
logy.

Functional measurements

The animals were placed in metabolic cages every 4 
weeks for 24-hour urinary specimens. Proteinuria was 
measured by the Bradford method (BioRad, Hercules, 
CA, USA). Systolic blood pressure was determined un-
der light ether anesthesia every 4 weeks by the tail-cuff 
method.

Histology

For semiquantitation of renal tissue damage, paraaffin-
embedded sections were stained using the Azan-Mallory 
method. The extent of interstitial and glomerular dam-
age was scored semiquantitatively from 0 to +3 by an 
investigator unaware of the origin of the slide (J.J.). At 
least five high-power fields were counted per section. 

Staining for proliferating cell nuclear antigen (PCNA) 
was performed on cryosections using a murine mono-
clonal antibody (MO8779, Dako, Hamburg, Germany). 
PCNA-positive cells were counted in at least 10 high-
power fields per section. MHC expression was assayed 
by staining cryosections using a murine antibody against 
RT1Bu (OX-3, Biosource, Fleurus, Belgium). MHC ex-
pression was quantitated for at least 10 high-power fields 
with the use of computer-based software (Meta View, 
Universal Imaging, West Chester, PA, USA) as de-
scribed previously [15].

RNA isolation and reverse transcription-polymerase 
chain reaction (RT-PCR)

Approximately 100 mg of renal tissue was homoge-
nized in 4 mol/L guanidine isothiocyanate (GITC) (Gibco, 
BRL, Eggenstein, Germany). RNA was extracted by 
ultracentrifugation through 5.7 mol/L caesium chloride 
(Gibco, BRL), quantitated by determining optical den-
sity at 260 and 280 nm and precipitated in ethanol [16]. 

Equal amounts of RNA per animal and group were pooled 
to account for interindividual variations yielding one pool 
of RNA per group. Reverse transcription (RT) of 1 µg 
RNA into cDNA was performed using random hexamers 
and avian myeloblastosis virus (AMV) reverse transcrip-
tase (Promega, Serva, Heidelberg, Germany). cDNA (100 
ge) were amplified by polymerase chain reaction (PCR) 
in a total volume of 30 µL using 2.5 U Ampli-Taq DNA 
polymerase (Perkin-Elmer-Cetus, Emeryville, CA, USA), 
100 µmol/L deoxyadenosine triphosphate (dATP), deoxy-
cytidine triphosphate (dCTP), deoxyguanosine tripho-
osphate (dGTP), and 50 µmol/L deoxythymidine triphosphate 
(dTTP) (Boehringer Mannheim, Mannheim, Germany) 
and 0.5 µmol/L of each primer in 1 ×PCR buffer [20 
mmol/L Tris-HCl, pH 8.3; 50 mmol/L KCl; 2 to 4 mmol/L 


were performed using the internal and the 3' /H11032 /H23043 /H9252 /H11006 /H9262 /H11001 was largely increased in allografts of hypertensive animals at 3 and 4 months. Histologic expression was performed. After an initial round of 20 cycles using the primer, an additional round of 10 to 20 cycles was then determined for each mRNA target. Every PCR reaction was adjusted until there was exactly the same signal for GAPDH in each group. The kinetics of the PCR reaction was then determined for each mRNA target. The optimal number of cycles enabling comparison of PCR products in the linear phase of the reaction was carried out for each mRNA target separately. Every PCR reaction was performed at least three times to verify reproducibility of differences between groups.

For PDGF-B, TGF-β1, and VEGF, nested PCR was performed. After an initial round of 20 cycles using the 5' and 3' primer, an additional round of 10 to 20 cycles were performed using the internal and the 3' primer.

**Table 1.** Systolic blood pressure (mm Hg, mean ± SEM)

<table>
<thead>
<tr>
<th>Month</th>
<th>Isograft</th>
<th>Isograft + hypertension</th>
<th>Allograft</th>
<th>Allograft + hypertension</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>85 ± 5&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>180 ± 15</td>
<td>95 ± 5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>180 ± 8</td>
</tr>
<tr>
<td>2</td>
<td>85 ± 9&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>180 ± 15</td>
<td>97 ± 9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>157 ± 16</td>
</tr>
<tr>
<td>3</td>
<td>86 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>190 ± 17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>121 ± 6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>145 ± 6</td>
</tr>
<tr>
<td>4</td>
<td>102 ± 6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>193 ± 9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>108 ± 6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>145 ± 6</td>
</tr>
<tr>
<td>5</td>
<td>102 ± 6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>213 ± 40</td>
<td>108 ± 6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>153 ± 3</td>
</tr>
<tr>
<td>6</td>
<td>92 ± 6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>148 ± 6</td>
<td>100 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>160 ± 9</td>
</tr>
</tbody>
</table>

<sup>a</sup>P < 0.05 vs. allograft + hypertension; <sup>b</sup>P < 0.05 vs. isograft + hypertension

**Table 2.** Proteinuria (mg/day, mean ± SEM)

<table>
<thead>
<tr>
<th>Month</th>
<th>Isograft</th>
<th>Isograft + hypertension</th>
<th>Allograft</th>
<th>Allograft + hypertension</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10 ± 2</td>
<td>10 ± 2</td>
<td>9 ± 1</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>2</td>
<td>8 ± 1</td>
<td>9 ± 1</td>
<td>19 ± 7</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>3</td>
<td>7 ± 1</td>
<td>14 ± 5</td>
<td>13 ± 4</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>4</td>
<td>11 ± 2</td>
<td>10 ± 1</td>
<td>27 ± 18</td>
<td>26 ± 7</td>
</tr>
<tr>
<td>5</td>
<td>19 ± 3</td>
<td>14 ± 1</td>
<td>47 ± 13</td>
<td>33 ± 4</td>
</tr>
<tr>
<td>6</td>
<td>11 ± 2</td>
<td>12 ± 2</td>
<td>61 ± 28</td>
<td>96 ± 51</td>
</tr>
</tbody>
</table>

<sup>a</sup>P < 0.01 for increase with time

**Statistical analysis**

Results are expressed as means ± SEM. Differences between groups were compared using unpaired analysis of variance (ANOVA) followed by Bonferroni testing (Instat, GraphPad Software). A P value of <0.05 was considered as significant.

**RESULTS**

**Functional measurements**

Blood pressure was significantly higher in DOCA/salt-treated animals compared to untreated animals (Table 1). Within DOCA/salt–treated animals, blood pressure was significantly higher in isografted animals compared to allografted animals at 3 and 4 months.

Proteinuria increased significantly over time in allografted animals, but not in isografted animals (Table 2). At 6 months, proteinuria was higher in DOCA/salt–treated allografted animals compared to DOCA/salt–treated isografted animals, but this difference did not reach significance. The serum creatinine was not significantly different between groups (Table 3) or compared to normal control rats (0.68 ± 0.04 mg/dL, N = 4).

**Histology**

In all animals, PCNA expressing could be observed. The number of PCNA-expressing cells/high-power field was higher at 3 months than at 6 months (Table 4). At 3 months, allografts of hypertensive animals contained significantly more PCNA-expressing cells compared to isografts and to allografts from normotensive animals (P < 0.05, Table 4). Most PCNA-expressing cells were tubular epithelial cells (Fig. 1), but glomerular mesangial cells also expressed PCNA.

Interstitial and glomerular fibrosis as determined by semiquantitative scoring from 0 to +3 was more pronounced in allografts of hypertensive animals compared to all other groups (Table 4). This difference was significant for both 3 and 6 months except for hypertensive animals at 6 months (Table 4).

In all groups, MHC expression was found to be higher at 3 months compared to 6 months. Histologic expression of MHC was largely increased in allografts of hypertensive animals compared to all other groups both at 3...
months and at 6 months (Table 4). The difference between allografts of hypertensive animals and the other groups was greater at 6 months than at 3 months. Several cell types expressed MHC, including glomerular cells, interstitial peritubular cells, and perivascular cells. MHC expression was most pronounced in the perivascular space while cells of the vascular wall itself did not express MHC (Fig. 2).

**Gene expression of MHC and growth factors**

MHC I transcription was readily detectable in isografts and in allografts, while transcription of MHC II was only observed in allografts (Fig. 2). When compared to isografts, MHC gene expression was increased in allografts from both normotensive and hypertensive animals (Fig. 2). Hypertension alone did not result in an increase of MHC transcription in isografts, but hypertension did augment transcription of MHC in allografts (Fig. 2). This effect was observed for both MHC I and MHC II, but to a greater extent for MHC II.

Gene expression of PDGF-B, TGF-β, VEGF, and collagen IV is shown in Figure 3. At 3 months, gene expression for PDGF-B was detected in isografts, but not in isografts. Treatment with DOCA/salt resulted in a marked increase in PDGF-B expression at 3 months. At 6 months, gene expression of PDGF-B was observed in all groups, was slightly increased in allografts compared to isografts, and was highest in allografts of hypertensive animals (Fig. 3). At 3 months, TGF-β expression could not be detected in normotensive isografts but in hypertensive isografts and in allografts. Similar to PDGF-B, treatment with DOCA/salt resulted in an increase in TGF-β expression at 3 months. At 6 months, TGF-β expression was also detectable in normotensive isografts, but to a lower degree than in the other three groups. Similar to the earlier time point, TGF-β expression was highest in allografts of hypertensive animals, but the difference between groups was less pronounced at 6 months compared to three months. Similar results were observed for collagen IV. Expression of VEGF was observed in all groups. VEGF was expressed to a greater extent in allografts compared to isografts at 3 months. Treatment with DOCA/salt had no influence on VEGF expression. At 6 months, expression of VEGF was similar in all groups.

| Table 3. Serum creatinine (mg/dL, mean ± SEM) |
|----------------|----------------|----------------|
| Month | Isograft | Isograft + hypertension | Allograft |
| 3 | 0.81 ± 0.04 | 0.72 ± 0.02 | 0.72 ± 0.02 |
| 6 | 0.8 ± 0.02 | 0.78 ± 0.06 | 0.76 ± 0.01 |

| Table 4. Immunohistologic expression of major histocompatibility complex (MHC) (arbitrary units), proliferating cell nuclear antigen (PCNA) (positive cells/high-power field) and interstitial fibrosis score (mean ± SEM) |
|----------------|----------------|----------------|
| MHC II | Isograft | Isograft + hypertension | Allograft |
| 3 months | 4460 ± 1132a | 4819 ± 1790a | 7461 ± 1337 |
| 6 months | 811 ± 288a | 3200 ± 1496a | 2560 ± 522a |
| PCNA | 3 months | 1.5 ± 0.2a | 1.07 ± 0.2a | 1.1 ± 0.2a |
| 6 months | 0.46 ± 0.12 | 0.81 ± 0.12 | 0.46 ± 0.12 |
| Fibrosis | 3 months | 1.3 ± 0.1 | 0.9 ± 0.2a | 1.1 ± 0.1a |
| 6 months | 1.0 ± 0.2a | 1.1 ± 0.2 | 1.0 ± 0.1a |

*P < 0.05 vs. allograft + hypertension

**DISCUSSION**

Several clinical studies confirmed an association between hypertension and subsequent chronic rejection [7–9]. Our data suggest that alloantigen-dependent and alloantigen-independent factors share final pathways in the pathogenesis of chronic allograft nephropathy. Initiation of the immune response with subsequent induction of growth factors, traditionally considered to be caused by alloantigen-dependent factors such as acute rejection, may also be part of the pathogenesis of graft injury by alloantigen-independent factors such as hypertension. Hypertension may act in addition to or synergistically with alloantigen-dependent factors on inflammatory pathways leading to graft injury.

The observed augmenting effect of DOCA/salt treatment on MHC expression in renal allografts is a new observation that to our knowledge has not been described before. The mechanism by which hypertension augments the expression of MHC in allografts is not fully understood. A direct effect of the substance DOCA itself or of possibly contaminating endotoxin cannot be excluded. For instance, DOCA/salt treatment increases blood pressure as well as fibrosis and TGF-β expression in the heart [25, 26], but MHC expression was not reported in these studies. However, the distribution of MHC II in the perivascular space indicates a direct pressure effect, possibly involving fluid shear stress. In vitro, induction of MHC antigens by shear stress has been reported in endothelial cells [27]. Abumiya et al [28] reported that in native kidneys of spontaneous hypertensive rats, accumulation of MHC II–positive macrophages could be observed. Similar to our observations, these MHC II–positive macrophages were predominantly located along the perivascular space. Interestingly, in the present study DOCA/salt treatment did not induce MHC in isografts, whereas it readily augmented MHC expression in allografts. The fact that MHC expression was not observed in isografts of hypertensive animals indicates...
that hypertension alone is not sufficient to induce MHC in this model. Allotransplantation appears to be a prerequisite for MHC expression. There appear to be additive or synergistic effects of hypertension and alloantigen-dependent mechanisms on MHC expression. Thus, hypertension may increase the immunogenicity of renal allografts, predisposing the graft to immunologic injury and the development of acute rejection episodes. Recently, Cosio et al [29] reported that hypertensive patients are at increased risk even for acute rejection episodes. The authors speculated that conditions of the graft (ischemia, delayed graft function) predisposing to acute rejections also lead to elevated blood pressure. The present study indicates that, on the other hand, hypertension may augment expression of MHC and growth factors and therefore predispose for acute rejection. However, the present study is limited because we did not provide a group of DOCA/salt–treated animals receiving antihypertensives. The demonstration that lowering blood pressure in DOCA/salt–treated rats reduces MHC and growth factor expression and ameliorates chronic allograft nephropathy would substantiate the causal link between hypertension and acute rejections of renal allografts.

One of the hallmarks of chronic rejection is the development of smooth muscle cell proliferation resulting in an extensive neointima and obliteration of the vessel lumen. One of the key mediators of smooth muscle cell proliferation is PDGF. PDGF, along with TGF-β, interleukin-1 (IL-1), and insulin-like growth factor (IGF) is mitogenic for smooth muscle cells and is involved in the vascular response to injury. PDGF is induced by hypertension in spontaneously hypertensive [30] and in DOCA-treated [31] rats, as well as by ischemia [32]. PDGF could also be identified in experimental models of acute and chronic rejection. Alpers et al [33] observed
PDGF-B chain in infiltrating monocytes within the rejecting arteries, similar to its localization in infiltrating monocytes in human atherosclerosis. PDGF expression has also been described in rejecting human allografts [34–36]. Thus, PDGF may have stimulatory effects on smooth muscle cells in an autocrine and/or paracrine manner to promote further intimal expansion and lesion progression to chronic rejection. In the context of these studies, it seems possible that PDGF expression is induced by the vascular injuries during acute rejection and augmented by hypertension starting the molecular events that link acute and chronic rejection. The fact that the effect of DOCA was not observed in isografts but was restricted to allografts indicated that allografts are particularly vulnerable to the detrimental influence of hypertension.

It should be noted that no animal model is able to fully reflect the clinical situation. The two animal strains used are genetically related and differ in only two MHC-I antigens and several non-MHC-related genes. Thus, no severe acute rejections occur, but without immunosuppression, Lewis animals transplanted from Fisher donors die within 30 days and the histology of the graft mainly shows acute rejection [37]. Immunosuppression diminishes or prevents these acute rejections enabling long-term survival. Although the Fisher-Lewis model is currently the only well-established model for chronic rejection, it is limited since it cannot reproduce the clinical picture in every aspect.

It is possible that antihypertensive treatment may reduce the development of chronic allograft nephropathy. This hypothesis is supported by studies in animals showing that blockers of the action of angiotensin II protects from chronic rejection [38, 39] but clinical data are scarce. Rahn et al [40] demonstrated that treatment with nitrendipine results in better renal function after 2 years, but this study included only 103 patients and was too short to assess long-term benefits on chronic allograft nephropathy. Until clinical studies are performed, it is necessary to emphasize the importance of controlling hypertension, maybe even more so after than before transplantation.

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Reprint requests to Ralf Schindler, M.D., Department of Nephrology, Universitätsklinikum Charité, Campus Virchow Klinikum, Augustenburger Platz 1, 13353 Berlin, Germany.

E-mail: ralf.schindler@charite.de

REFERENCES


