Advancements in renal protection
Waanders, Femke

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2008

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
CHAPTER 4

SPIRONOLACTONE AMELIORATES FOCAL GLOMERULOSCLEROSIS AND TRANSPLANT ARTERIOPATHY IN EXPERIMENTAL CHRONIC ALLOGRAFT NEPHROPATHY IN RATS

Femke Waanders1*, Heleen Rienstra2*, Jan Rozing2, Annemieke Smit-van Oosten4, André Zandvoort2,4, Gerjan Navis1, Harry van Goor3 and Jan-Luuk Hillebrands2

1 Department of Nephrology, 2 Department of Cell Biology, 3 Department of Pathology and Medical Biology, 4 Central Animal Facility, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands

* Contributed equally

In progress
Abstract

Background. Chronic allograft nephropathy (CAN) is the leading cause of long-term renal allograft loss, for which no effective therapy is available. Ischemia/reperfusion injury at transplantation predisposes to development of CAN. Increasing evidence indicates that aldosterone is a direct mediator of renal damage via the mineralocorticoid receptor (MR). The aldosterone antagonist spironolactone attenuates renal ischemia/reperfusion injury in rats. Thus, aldosterone blockade might protect against CAN. In this study we determined the efficacy of spironolactone in attenuating CAN after experimental renal transplantation in rats.

Methods. Renal transplantation was performed in the allogeneic Dark Agouti-to-Wistar Furth rat strain combination. Dark Agouti-to-Dark Agouti isografts served as controls. Recipients received either spironolactone (20 mg/kg BW) (+) or vehicle (-) daily by oral gavage until the end of the experiment. Treatment started 2 days prior to transplantation. 4 experimental groups were included: 1) allograft- (n=9), 2) allograft+ (n=9), 3) isograft- (n=8) and 4) isograft+ (n=8). Before and every 2 weeks after transplantation systolic blood pressure and urinary protein excretion were determined. Grafts were retrieved 12 weeks after transplantation and processed for histological analyses (focal glomerulosclerosis (FGS), interstitial fibrosis (IF), transplant arteriopathy (TA) and macrophage influx).

Results. Isograft recipients did not develop proteinuria. In contrast, allograft recipients developed clear proteinuria which was attenuated by spironolactone (77±28 mg/day [group 1] vs. 41±10 mg/day [group 2]; p=0.06). In time, spironolactone did not affect systolic blood pressure. In contrast to isografts, allografts presented with prominent FGS and IF. Spironolactone significantly attenuated the severity of FGS in allografts (100±37 A.U. [group 1] vs 47±17 A.U. [group 2], p<0.05) without affecting IF. Reduced FGS in the allografts treated with spironolactone was accompanied by reduced glomerular macrophage influx (p<0.05, group 1 vs. group 2). Allografts developed TA which was significantly ameliorated (p<0.05) by spironolactone.

Conclusion. MR blockade by the aldosterone antagonist spironolactone ameliorates the development of proteinuria, FGS and TA in an experimental rat model for CAN, suggesting a role for aldosterone in the pathogenesis of CAN. We propose that the observed glomerulo- and vasoprotective effects of spironolactone might result in improved long-term graft survival.
Introduction

Over the last decades, the introduction of new immunosuppressive drugs, combined with better preservation techniques has markedly reduced the incidence of acute rejection after renal transplantation.1-3 Short-term graft survival has therefore increased, but unfortunately, no improvements have been obtained regarding long-term chronic transplant dysfunction.4 Consequently, the main cause of graft loss is chronic allograft nephropathy (CAN) characterized by a progressive loss of renal function in combination with histological alterations such as glomerulopathy, transplant arteriopathy (TA), tubular atrophy, and interstitial fibrosis (IF). The development of CAN is a multifactorial process influenced by both alloantigen-dependent and independent factors.4 Alloantigen independent risk factors for the development of CAN include ischemia/reperfusion injury at transplantation, delayed graft function, donor age, donor/recipient co-morbidity such as hypertension and nephrotoxicity of calcineurin inhibitors.5-8 So far, no effective treatment is available to attenuate CAN.

There is increasing evidence that the mineralocorticoid hormone aldosterone is directly involved in the development and progression of renal disease via non-epithelial mineralocorticoid receptors (MR).9-12 In vitro studies have confirmed that the pathophysiological effects of aldosterone derive at least in part from its non-hemodynamic actions. Aldosterone increases the production of TGF-β, reactive oxygen species and PAI-1 which can be abolished by MR blockade.13-16 Moreover, aldosterone induces collagen gene expression and synthesis in vascular smooth muscle cells, which can also be prevented by MR blockade.17 Animal studies have shed more light on the pathophysiological non-epithelial mediated effects of aldosterone and the MR. In several models of nephropathy including spontaneously hypertensive stroke-prone rats MR antagonists markedly ameliorated glomerular and/or tubulo-interstitial injury without effects on systemic blood pressure or volume status.12,18

Recently, it was shown that MR blockade by spironolactone prior to the induction of renal ischemia/reperfusion injury in rats prevented renal function loss, proteinuria and oxidative stress.19 Moreover, spironolactone increased survival and prevented the progression of tubulo-interstitial fibrosis and vasculopathy in cyclosporine nephrotoxicity in rats.20 Since ischemia/reperfusion injury, nephrotoxicity of calcineurin inhibitors interstitial renal damage and vasculopathy (i.e. transplant arteriopathy) play an important role in the de-
development and progression of CAN, we hypothesize that aldosterone receptor blockade could have beneficial effects in CAN. To test this hypothesis, we determined the efficacy of MR blockade by spironolactone in attenuating CAN after experimental renal transplantation in rats.

Materials and methods

Animals, treatment and surgical procedures
Fortytwo inbred adult male rats were studied. Rats were housed in a temperature-controlled room of 18-20 °C with a 12h-light/dark cycle. The rats had free access to standard chow and drinking water. The local animal ethics committee at the University of Groningen approved all experimental procedures and the Principles of Laboratory Animal Care (NIH publication no. 85-23) were followed.

Treatment started two days prior to transplantation in both donor and recipient. Spironolactone (Sigma-Aldrich S3378) was dissolved in 1% 2-hydroxyethyl cellulose (Sigma-Aldrich 308633) and administered daily in a dose of 20 mg/kg bodyweight by oral gavage, based on previous studies in ischemia/reperfusion19 and cyclosporine nephrotoxicity20 in Wistar rats. Vehicle-treated rats received corresponding volumes of the solvent by daily oral gavage.

Renal transplantation was performed according to standard procedures.21 For the allogeneic transplantations, male Wistar Furth rats (WF; Charles River laboratory) served as recipients and for the isograft transplantations male Dark Agouti rats (DA; Harlan, Zeist, the Netherlands). At the time of transplantation the WF allografts weighed 264±5 g and the DA isografts 251±2 g, without differences between the groups. Female DA rats, weighing 183±1 g with kidneys weighing 0.7 ± 0.0 g served as donors for all transplantations (both the allografts and the isografts). Donor rats were anaesthetized with isoflurane/O2, left kidneys were flushed in situ with saline and removed after which donor rats were sacrificed. Kidneys were preserved in saline on ice for 20 minutes and transplanted orthotopically into the recipient rat under isoflurane/O2 anaesthesia. The left renal vessels and ureter were anastomosed end-to-end using 10-0 prolene sutures. Vascular clamps were released after a standardized warm ischemia time of 25 minutes. All rats were given buprenorfine (Temgesic®) 0.01 mg/kg subcutaneously during transplantation, 8-10 h after and one day after transplantation for pain relief. To prevent acute rejection, Cyclosporine A (CsA; 5 mg/kg/d, Sandimmune, Sandoz Pharma AG, Basel, Switzerland) was daily ad-
ministered subcutaneously to all rats for ten days. Fourteen days after transplantation, the native right kidney was removed and the transplanted kidney was inspected for viability and hydronephrosis. During nephrectomy, 8-10 h after and one day after nephrectomy rats also received buprenorin in a concentration of 0.01 mg/kg. Eight animals ($n=6$ DA to WF allografts and $n=2$ DA to DA isografts) in whom surgical (ischemia, $n=4$; iatrogenic intestinal perforation, $n=1$) or urological complications (hydronephrosis, $n=3$) were observed, were sacrificed at nephrectomy and excluded from follow-up.

Four animals were sacrificed prematurely (see results section: survival). Accordingly, for the long-term follow-up the following groups were studied: $n=6$ DA to WF allografts treated with vehicle, $n=8$ DA to WF allografts treated with spironolactone, $n=8$ DA to DA isograft controls treated with vehicle and $n=8$ DA to DA isografts treated with spironolactone. Rats were sacrificed 12 weeks after renal transplantation.

Clinical parameters
To determine spironolactone and vehicle dosage, body weight was measured daily. Systolic blood pressure was measured non-invasively every other week. Rats were trained for two weeks, prior to renal transplantation to undergo blood pressure measurements. A multi-channel computerized system was used with tail cuffs and photoelectric sensors to detect the tail pulse (CODA$^\text{TM}$; Kent Scientific Corporation, Torrington, CT, USA). Rats were placed in restrainers while temperature of the tail was maintained at 35 to 37ºC. For each rat, the value was calculated from the mean of three to five consecutive measurements.

After blood pressure was measured, rats were placed in individual metabolic cages (Bioquant$^\text{TM}$; Merck, Darmstadt, Germany) to collect 24 hour urine samples. In these metabolic cages rats were not allowed to eat, but they had free access to water. Fasting blood samples were taken prior to transplantation and at the end of the study. Urinary and serum concentrations of protein, creatinine, urea, sodium and potassium were all analyzed on a multi-test analyzer system (Roche Modular; F. Hoffmann-La Roche Ltd, Basel, Switzerland).

Sacrification, assessment of renal morphologic damage and transplant arteriopathy
At the end of the study (week 12), rats were anaesthetized, kidneys were perfused with saline and rats were sacrificed. A coronal tissue slice through the midportion of the kidney was fixed in 4% formaldehyde and processed for paraffin embedding. Paraffin embedded sections (4μm) were stained with periodic acid-Schiff (PAS) and Verhoeff to evaluate...
focal glomerulosclerosis (FGS), interstitial fibrosis (IF) and transplant arteriopathy (TA). To assess the degree of FGS, a qualified, independent pathologist semi-quantitatively scored 50 glomeruli on a scale of 0 to 4 by light microscopy in a blinded fashion. FGS was scored as present when collapse of capillary lumens, mesangial matrix expansion, hyalinosis, and adhesion formation were present in the same quadrant. If 25% of the glomerulus was affected, a score of 1 was given, 50% was scored as 2, 75% as 3 and 100% as 4. The degree of IF was scored similarly in 30 consecutive visual fields. IF was defined as expansion of the interstitial space, with or without the presence of atrophied and dilated tubules and thickened tubular basement membranes. Medullary tissue, glomeruli and vessels were excluded from the calculated areas of fibrotic involvement. A score of 0 was given when no interstitial fibrosis was present in a field, 1 for 0-25% with IF, 2 for 25-50%, 3 for 50-75% and 4 for 75-100% of the field showing IF. To obtain the final score, we multiplied the degree of injury by the percentage of glomeruli (for FGS) or visual fields (for IF) with the same degree of injury and added these scores, rendering a theoretical range of 0 to 400.


The presence of TA was assessed in all elastin-positive arteries with a diameter wider than 120 μm and a width to length ratio of at least 1:3 (longitudinally cut arteries were excluded). The number of arteries with TA as indicated by the presence of neointima formation, were expressed as a percentage of the total amount of vessels that met the aforementioned criteria. Moreover, the severity of intraluminal narrowing was expressed as percentage occlusion of the lumen measured in a blinded fashion using computerized image analysis (Advanced QUIPS, Leica Imaging Systems, Cambridge, UK).

Renal macrophage influx
Deparaffinized and rehydrated sections (4 μm) were subjected to heat-induced antigen retrieval by overnight incubation in a 0.1 M Tris/HCl buffer (pH 9.0) at 80°C. Endogenous peroxidase was blocked with 0.3% H2O2 in phosphate-buffered saline (PBS) for 30 minutes and sections were incubated with an ED1-antibody (1:750; Serotec, Oxford, UK) for 60 minutes at room temperature. Binding of the antibody was detected using sequential incubations with peroxidase (PO)-labelled rabbit anti-mouse and PO-labelled goat anti-rabbit antibodies; both for 30 min. Peroxidase activity was developed using 3,3’-diaminobenzidine tetrachloride (DAB) for 10 min. Sections were counterstained with haematoxylin. Macrophages were assessed in 40 glomeruli and in 30 interstitial fields per kidney.

Data analysis
Data are expressed as mean ± standard error. Statistical analysis of group differences was per-
formed by a Kruskal-Wallis ANOVA on ranks. Correlations between proteinuria and the parameters FGS and glomerular macrophages were analyzed using Pearson’s correlation coefficient. Statistical analyses were performed using SPSS version 14.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was assumed at the 5% level (two-tailed).

Results

Survival

We had to prematurely sacrifice three animals in the allograft group treated with vehicle (on day 40, 49 and 51 after transplantation) and one animal in the allograft group treated with spironolactone (on day 51). In all cases the reason for sacrifice was a declined condition, pilo-erected fur and weight loss. After histological examination it appeared that all four animals had severe TA. The 3 vehicle-treated allografts had a mean intraluminal narrowing of 50±11% versus 22±10% in the survivors of that group. The prematurely sacrificed spironolactone-treated allograft had 40% intraluminal narrowing versus 10±4% in the survivors of that group.

Almost all arteries from the prematurely sacrificed animals were affected by neointima formation. All animals in the isograft control groups survived until the end of the experiment (12 weeks). The survival curves reached a borderline significant difference (Chi square=6.437, p=0.09, Figure 1). The prematurely sacrificed animals were excluded from all analyses.

![Figure 1](image-url)

Figure 1. In the allograft group treated with vehicle three animals had to be prematurely sacrificed (on day 40, 49 and 51 after transplantation) and in the allograft group treated with spironolactone one animal (on day 51). In all cases the animals suffered from histological proven CAN with TA. All animals in the isograft control groups survived until the end of the experiment. The survival curves reached a borderline significant difference (Chi square=6.437, p=0.09).
Body weight
At the time of transplantation, there were no significant differences in body weight between the groups. From day 4 until the end of the experiment the allografts had a significantly higher bodyweight than the isografts (growth curves are shown in Figure 2). Spironolactone had no significant effect on body weight at any time point. However, at sacrifice the spironolactone-treated allografts had a lower bodyweight of borderline significance ($p=0.07$) compared to the vehicle-treated allografts.

![Figure 2](image_url)

**Figure 2.** Growth curves of the rats during the study. From day 4 until the end of the experiment the allografts had a higher bodyweight than the isografts. There were no differences in body weight between the vehicle and the spironolactone-treated rats at any time point. However, at sacrifice the spironolactone-treated allografts had a lower bodyweight of borderline significance ($p=0.07$) compared to the vehicle-treated allografts. *$p<0.05$ allografts versus isografts.*

Creatinine clearance, urea and electrolytes
Prior to transplantation, creatinine clearances, urea levels, and electrolytes were similar in the WF and DA rats (data not shown). At termination, the allografts had a significantly lower creatinine clearance, without significant effects of spironolactone (Figure 4A). Urea levels were significantly higher in the allografts compared to the isograft controls. In the allografts spironolactone significantly increased serum urea levels and urinary volume production, without having effects in the isograft controls (Table 1). Urinary sodium excretion tended to increase in the spironolactone-treated allografts compared to the vehicle-treated allografts (Table 1). At termination no significant differences in serum sodium and potassium levels were observed between the groups (Table 1). Urinary potas-
sium excretion was significantly higher in the allografts versus the isografts, without effects of spironolactone (Table 1).

### Table 1. Clinical parameters at the end of the study. All values are expressed as mean ± SE. *p<0.05 versus all other groups, #p<0.05 versus both isograft controls (vehicle- and spironolactone-treated), †p<0.05 versus the isograft control on the same treatment; ‡ the difference in proteinuria between vehicle and spironolactone treated allografts was of borderline significance (p=0.06).

<table>
<thead>
<tr>
<th></th>
<th>Allograft</th>
<th>Isograft controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>Spironolactone</td>
</tr>
<tr>
<td></td>
<td>(n=6)</td>
<td>(n=8)</td>
</tr>
<tr>
<td>Serum urea (mmol/L)</td>
<td>18 ± 2 *</td>
<td>32 ± 5 *</td>
</tr>
<tr>
<td>Serum sodium (mmol/L)</td>
<td>143 ± 1.2</td>
<td>143 ± 0.4</td>
</tr>
<tr>
<td>Serum potassium (mmol/L)</td>
<td>4.7 ± 0.3</td>
<td>4.8 ± 0.1</td>
</tr>
<tr>
<td>Urinary volume (mL)</td>
<td>19 ± 2</td>
<td>27 ± 3 *</td>
</tr>
<tr>
<td>Proteinuria (mg/d)</td>
<td>77 ± 28</td>
<td>41 ± 10 *</td>
</tr>
<tr>
<td>Urinary sodium (mmol/d)</td>
<td>0.85 ± 0.18 †</td>
<td>1.01 ± 0.15 ‡</td>
</tr>
<tr>
<td>Urinary potassium (mmol/d)</td>
<td>1.43 ± 0.13 ‡</td>
<td>1.45 ± 0.08 ‡</td>
</tr>
</tbody>
</table>

Urinary Protein Excretion and Blood pressure

In the vehicle-treated allografts a significant increase in urinary protein excretion was observed during the 12 weeks of the experiment, which was significantly ameliorated by spironolactone (the percentage change is shown in Figure 3A). The difference in absolute urinary protein excretion between the vehicle and the spironolactone treated allografts at the end of the experiment was of borderline significance (p=0.05, Table 1). In isograft controls urinary protein excretion remained at baseline levels; this was unaffected by spironolactone (Figure 3A, Table 1).

During the 12 weeks of the experiment systolic blood pressure gradually increased in the allografts, without effects of spironolactone (Figure 3B). Since the DA rat strain was difficult to handle, we were unable to measure blood pressure reliably in these rats due to stress-related effects on blood pressure.

Kidney weight and renal structural changes

Vehicle-treated allografts had the highest increase in kidney weight compared to all other groups (absolute kidney weights are show in Figure 4B). FGS was abundantly present in the allografts, which was significantly ameliorated by spironolactone (Figure 4C, photomicrographs are presented in Figure 5A). IF was signif-
spironolactone (Figure 4D).

Figure 3. Time course of proteinuria development and blood pressure values in vehicle- and spironolactone-treated allografts and isografts. Data are expressed as mean ± SE. Abbreviation: Tx, renal transplantation.

A) In the vehicle-treated allografts, a significant increase in proteinuria from baseline was observed during the 12 weeks of the experiment. The amelioration in urinary protein excretion by spironolactone in the allografts reached borderline significance (p=0.08) after 10 weeks of treatment and was significant after 12 weeks (p<0.05).

B) During the 12 weeks of the experiment, systolic blood pressure gradually increased in the allografts, without effects of spironolactone. Since the DA rat strain was difficult to handle, we were unable to measure blood pressure reliably in these rats due to stress-related effects on blood pressure.

*p<0.05 vs all other groups, #p<0.05 vs both isograft controls.
Figure 4. Graphs represent mean ± SE.
A) Creatinine clearance.
B) Kidney weight.
C) Focal glomerulosclerosis, semi-quantitative score (arbitrary units 0 to 400).
D) Interstitial fibrosis, semi-quantitative score (arbitrary units 0 to 400).
E) Percentage of TA affected vessels (presence of neointima formation).
F) Percentage intraluminal narrowing with all measured renal vessels pooled per group.
G) Mean glomerular macrophages (mean number per glomerulus of 40 glomeruli scored).

*p<0.05 vs all other groups, †p<0.05 vs both isograft controls, ‡p<0.05 vs the isograft control on the same treatment.
Transplant arteriopathy (TA)
Allografts presented with severe neointima formation, indicating development of TA in our experimental model for CAN (Figure 5B). The proportion of TA-affected vessels was significantly higher in the vehicle-treated allografts compared to the spironolactone-treated allografts, without differences between the isograft controls (Figure 4E). When all measured renal vessels were pooled per group, the percentage intraluminal narrowing in the vehicle-treated allografts was significantly higher compared to the spironolactone-treated allografts, without differences between the isograft controls (Figure 4F). There were no significant differences between the spironolactone-treated allografts and both isografted groups (Figure 4E and F).
Thus, not only the proportion of TA affected vessels was lower in the spironolactone treated allografts compared to the vehicle treated allografts, but the vasculopathy itself was also less severe.

Renal macrophage influx
The influx of macrophages in the glomeruli in vehicle-treated allografts was significantly higher compared to the spironolactone-treated allografts (Figure 4G, photomicrographs are presented in Figure 5C). Spironolactone did not affect interstitial macrophage influx (data not shown).

Proteinuria: correlation analyses with FGS, glomerular macrophages and renal function
To investigate whether the glomerular effects of spironolactone in this model for CAN can be monitored non-invasively by proteinuria, we performed a partial correlation analysis controlling for the different groups (Figure 6A, R=0.937, p<0.0001). On bivariate correlation analysis we only found a strong and significant correlation between proteinuria and FGS in the allografted animals treated with vehicle (R=0.967, p<0.01) or spironolactone (R=0.835, p<0.01), but not in the isografted animals without proteinuria or glomerular damage. There were no significant correlations between proteinuria and IF (data not shown).
We also investigated whether proteinuria was related to the glomerular influx of macrophages. Partial correlation analysis controlling for the different groups was statistically significant (Figure 6B, R=0.661, p<0.0001). However, this only depended on the allografted group treated with vehicle, since only in that group bivariate correlation analysis was significant (R=0.952, p<0.01), whereas this was not the case for the allografts treated with spironolactone or the isografts (Figure 6B).
Figure 5. Representative figures of histology. Abbreviations used in the pictures: allo, allograft; iso, isograft; -, treated with vehicle; +, treated with spironolactone.

A) Sections stained with periodic acid-Schiff. Focal glomerulosclerosis in vehicle-treated allografts was significantly ameliorated by spironolactone.

B) Renal vessels in sections stained with Verhoeff. The development of transplant arteriopathy (neointima formation) was significantly ameliorated by spironolactone.

C) Sections stained with an ED1 antibody. Glomerular influx of macrophages seen in vehicle treated allografts was significantly inhibited by spironolactone.
To investigate whether the effects of spironolactone on proteinuria were dependent on its effects on renal function, we performed a partial correlation analysis controlling for the different groups. Proteinuria was not correlated to creatinine clearance ($R = -0.226, p = 0.238$). Also when analyzing the groups separately on bivariate analysis, we did not observe a correlation between proteinuria and renal function.

**Discussion**

This is the first study that demonstrates glomerulo- and vasoprotective effects of the aldosterone antagonist spironolactone in experimental chronic allograft nephropathy (CAN).

As anticipated, CAN was characterized by renal function impairment, as manifested by reduced creatinine clearance, proteinuria, transplant arteriopathy (TA), focal glomerulosclerosis and interstitial fibrosis with inflammatory infiltrates and tubular atrophy. Aldosterone blockade by spironolactone ameliorated the development of proteinuria, glomerular macrophage influx, focal glomerulosclerosis and TA, suggesting a role for aldosterone in the pathogenesis of CAN. Moreover, the increase in kidney weight was
higher in the vehicle-treated allografts, suggesting that spironolactone attenuated renal hypertrophy. However, spironolactone had no effect on interstitial fibrosis and inflammation in this model. Spironolactone as such had no effect in the isograft controls without CAN, neither on renal function and protein excretion nor on morphology. The observed increased urea levels in combination with increased urine output and a trend towards a higher sodium excretion and a lower body weight in the spironolactone-treated allografted rats are indicative of a diuretic effect of spironolactone in this model. However, this effect was apparently without an effect on blood pressure. It is unlikely therefore that the beneficial effects of spironolactone are fully accounted for by its diuretic effects.

The development of TA was significantly ameliorated by spironolactone. Not only the proportion of TA-affected vessels was lower in the spironolactone-treated allografts compared to the vehicle-treated allografts, but also the severity of vascular narrowing was significantly reduced. It is of interest that the pathogenesis of CAN, especially TA, shares many features with the pathogenesis of systemic atherosclerosis. Known risk factors for cardiovascular disease, like hypertension, proteinuria and hypercholesterolemia are independent risk factors for CAN.22,23 A recent study showed that MR blockade prevented the development of atherosclerotic plaques by 70%, reduced oxidative stress as well as the inflammatory response in apolipoprotein E-deficient mice fed a high cholesterol diet.24 Also, aldosterone signaling pathways appear similar in both the kidney and the cardiovascular system. Aldosterone-induced signaling in vascular cells involves the MAP kinase pathway with participation of the epidermal growth factor receptor (EGFR). The MR uses the EGFR signaling pathway to stimulate ERK1/2 phosphorylation.25,26 In stroke prone SHR rats spironolactone reduced cerebral infarct size, which was associated with decreased EGFR expression.27 Recently, Nakamura et al. identified murine double-minute type 2 (MDM2) as one of the genes that regulates cell proliferation of cultured human vascular smooth muscle cells induced by MR-mediated aldosterone stimulation.28 Of note, MDM2 is a novel signaling molecule that participates in antiapoptosis and cell proliferation.

What could be the mechanism of the amelioration of glomerular macrophage influx by spironolactone? We found a correlation between proteinuria and glomerular macrophages only in the vehicle-treated allografts. The amelioration of glomerular macrophage influx by spironolactone was not correlated to proteinuria, and therefore probably not secondary to the antiproteinuric effect. Aldosterone is known to induce endothelial dysfunction
and decreased vascular compliance, effects that require a functioning MR. Moreover, aldosterone not only increases endothelial volume, but also facilitates intercellular gap formation, serving as a diffusion pathway. Since the MR is present in the preglomerular vasculature and in glomeruli, aldosterone antagonism by spironolactone might have direct effects on glomerular endothelium preventing the passing of macrophages and on glomerular capillary pressure.

There are several mechanisms by which spironolactone may have exerted its glomerulo- and vasoprotective effects in our model for CAN. First, spironolactone prevents renal ischemia/reperfusion injury, which predisposes the kidney at the time of transplantation to the development of CAN. Second, spironolactone attenuates cyclosporine nephrotoxicity in rats. It prevents the well-known effect of cyclosporine inducing renal vasoconstriction and impairing renal blood flow. Nephrotoxicity of calcineurin inhibitors is an important risk factor for the development of CAN. However the combination of alloantigen-dependent factors with ischemia/reperfusion injury and cyclosporine nephrotoxicity is a prerequisite for later development of CAN. In the isograft controls ischemia/reperfusion and ten days of cyclosporine treatment did not induce renal damage. Third, MR blockade by spironolactone may have prevented chronic profibrotic effects of aldosterone on the kidney. Aldosterone is known to increase the production of TGF-β, PAI-1, reactive oxygen species and collagen, which can be abolished by MR blockade. Finally, the requirement for a concomitant high sodium diet in most animal models of aldosterone-related renal injury and the absence of tissue injury in forms of secondary aldosteronism without hypertension, suggest that aldosterone’s ability to increase blood pressure and intraglomerular pressure is at least a major determinant of kidney damage and that the diuretic effects of spironolactone may be involved in its renoprotective effect. However, in most animal and human studies the renoprotective effects of MR blockade by spironolactone were independent of effects on blood pressure or volume status. Our study was not specifically designed to elucidate the mechanisms by which spironolactone exerted its renoprotective effects, but several inferences can be made. We did not observe effects on blood pressure, but the fact that spironolactone increased urea levels in combination with increased urine output and a trend towards a higher sodium excretion and a lower body weight indicate that spironolactone acted as a diuretic, blocking the effects of aldosterone on volume/sodium status. Apart from these effects, blockade of the nonhemodynamic profibrotic actions of aldosterone by spironolactone likely played a major role in attenuating neointima formation and glomerular fibrosis.
In spite of the protective effects on the glomerulus and the vascular abnormalities, spironolactone did not affect the interstitial abnormalities of CAN. This suggests that in this model the pathogenesis of the glomerular, vascular and interstitial abnormalities, respectively, is not uniform. This is in line with other studies from our group in this model, showing that endothelial cells in glomerular and peritubular capillaries are partly derived from the recipient, whereas endothelial cells in larger veins, arterioles and arteries (also the arteries with transplant arteriopathy) are derived from the donor, indicating endothelial diversity in the renal vasculature (Rienstra et al., submitted). The absence of an effect of spironolactone on the interstitial abnormalities in this model is remarkable, as, first, it dissociates from the protective effects on the glomerular and the vascular abnormalities of CAN and, second, in other models of renal disease, for instance adriamycin-induced proteinuria, we found a trend towards protective effects on the interstitial abnormalities with spironolactone treatment as well.38

To investigate whether the observed glomeruloprotective effects of spironolactone in this model for CAN can be monitored non-invasively by proteinuria, we performed correlation analyses. Indeed, proteinuria was significantly related to glomerulosclerosis, but not to interstitial fibrosis. In contrast to proteinuria, the glomeruloprotective effects of spironolactone were not related to renal function. This is in contrast to a recent randomized placebo-controlled trial in type 2 diabetic patients with overt nephropathy which showed that the decline in albuminuria by the addition of spironolactone to either an ACE inhibitor or an AT1 receptor blocker was significantly related to a decrease in cGFR.39 The authors hypothesize that the antiproteinuric effect of spironolactone found in their study was due to a possible decreased angiotensin II sensitivity which could have resulted in decreased intraglomerular pressure. In our study, the spironolactone-treated allografts had a lower creatinine clearance with significantly increased urea levels. This could indicate that spironolactone decreased intraglomerular pressure and thereby glomerular filtration, explaining the combination of the lower creatinine clearance, and the reduction in proteinuria and FGS. In vitro data showing that aldosterone inhibits vasoconstriction in rabbit microdissected perfused afferent arterioles, which is abolished by spironolactone, would be consistent with this assumption.31 However, we did not measure renal hemodynamics, so this assumption remains to be proven.

We found a trend towards a survival benefit in the spironolactone-treated allografts compared to the vehicle group, which is explained by increased graft loss in the vehicle group.
due to the development of CAN with severe transplant arteriopathy. This is in line with data from experimental cyclosporine-induced renal damage, in which a significantly increased rat survival was observed during treatment with spironolactone.20

Currently, on the long-term many renal grafts are lost due to CAN. Aldosterone receptor blockade has little side effects compared to standard immunosuppressive agents that are currently subscribed to renal transplant patients. The pathogenesis of CAN is complex, involving multiple pathways. Our data show that MR blockade by spironolactone has selective protective effects on glomerular and vascular damage in an experimental rat model for CAN. We propose that spironolactone might be a useful adjunct therapy to attenuate CAN and improve long-term graft survival.

Acknowledgements

This study was supported by a Career Stimulation Program Grant from the Dutch Kidney Foundation (C03.6015) to J.L.H. and the Jan Kornelis de Cock Foundation. We would like to thank Marian Bulthuis and Michel Weij for skilled (bio)technical assistance. None of the authors have involvements that might raise the question of bias in the work reported nor in the conclusions, implications or opinions stated.
References


