Spironolactone ameliorates transplant vasculopathy in renal chronic transplant dysfunction in rats

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OVER THE LAST DECADES, THE introduction of new immunosuppressive drugs, combined with better preservation techniques, has significantly reduced the incidence of acute rejection after renal transplantation, resulting in markedly increased short-term graft survival (3, 18, 26). Unfortunately, no improvements have been obtained in long-term graft survival. Consequently, the main cause of graft loss, second to patient death with a functioning graft, is chronic transplant dysfunction (CTD). CTD is characterized by a progressive loss of renal function coinciding with chronic histopathological lesions. These lesions include transplant vasculopathy (TV), interstitial fibrosis (IF), tubular atrophy, and glomerulosclerosis (7, 8, 35, 44). TV is characterized by intimal proliferation of smooth muscle cells and luminal occlusion (i.e., neointima formation) (20). So far, no effective treatment is available to ameliorate these histopathological lesions characteristic of CTD. The pathogenesis of CTD is most likely multifactorial.

A novel potential approach for intervention in CTD is mineralocorticoid receptor (MR) blockade. The MR is the receptor for the mineralocorticoid hormone aldosterone. There is increasing evidence that aldosterone is directly involved in the development and progression of renal disease via interaction with a nonepithelial MR, independent of its hemodynamic effects (1, 10, 15, 36, 39). In several animal models of native kidney disease, MR antagonists markedly ameliorated renal injury (6, 14, 16, 17, 34, 38, 45). Furthermore, MR blockade reduced neointima formation after coronary angioplasty and stenting in pigs, demonstrating vasculoprotective potential (47, 48), and the MR antagonist spironolactone was shown to protect against renal ischemia-reperfusion injury when given before induction (29). Taken together, these results fuel the hypothesis that MR blockade by spironolactone can ameliorate CTD with TV. To test this hypothesis, we analyzed the efficacy of spironolactone in a well-established rat model for renal chronic transplant dysfunction (25).

MATERIALS AND METHODS

Animals, treatment, and surgical procedures. Forty-two inbred adult male rats were studied. Rats were housed in a temperature-controlled room with a 12:12-h light-dark cycle. 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 (National Institutes of Health publication no. 85-23) were followed.

Treatment started 2 days before transplantation in both donors and recipients. Spironolactone (S3378; Sigma-Aldrich) was dissolved in 1% 2-hydroxyethyl cellulose (308633; Sigma-Aldrich) and administered daily at a dose of 20 mg/kg body wt by oral gavage, based on previous studies performed in rat models of experimental ischemia-reperfusion injury (29) and cyclosporine nephrotoxicity (33). Vehicle-treated rats received corresponding volumes of 1% 2-hydroxyethyl cellulose without spironolactone by daily oral gavage.

Renal transplantation was performed in the female Dark Agouti (DA)-to-male Wistar-Furth (WF; allograft) and female DA-to-male DA (isograft) strain combinations according to standard procedures (43). DA and WF rats were obtained from Harlan Nederland (Zeist, The Netherlands) and Charles River Nederland (Maastricht, The Netherlands), respectively. At the time of transplantation, the weight of WF and DA recipients was 264 ± 5 and 251 ± 2 g, respectively. At the time of transplantation, the weight of DA donors was 183 ± 1 g, providing kidney grafts weighing 0.7 ± 0.0 g. Donor rats were anesthetized with isoflurane/O2, and left kidneys were flushed in situ with saline and removed. The donor rats were then killed. Kidneys

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were preserved in ice-cold saline for 20 min and then orthotopically transplanted into the recipient from which the left kidney was removed under isoflurane/O_2 anesthesia. The graft renal vein, artery, and ureter were anastomosed end to end to the recipients' left renal vein, artery, and ureter, respectively, using 10-0 prolene sutures. Vascular clamps were released after a standardized warm ischemia time of 25 min. Both adrenal glands of the recipient remained in situ. All transplanted rats were given buprenorphine (Temgesic; 0.01 mg/kg) subcutaneously for 8–10 h and 24 h after transplantation for pain relief. To prevent acute rejection, cyclosporin A (5 mg/kg/day; Sandimmune; Sandoz Pharma, Basel, Switzerland) was administered subcutaneously to all transplanted rats daily for 10 days. Fourteen days after transplantation, the native right kidney was removed and the transplanted kidney was inspected for viability and hydrenephrosis. For 8–10 h and 24 h after nephrectomy, rats received buprenorphine (0.01 mg/kg) subcutaneously. Eight rats (6 allograft and 2 isograft recipients) in which surgical or urological complications were observed were killed at nephrectomy and excluded from follow-up.

Clinical variables. To determine spironolactone and vehicle dosage, body weight was measured daily. Systolic blood pressure was measured noninvasively before and every other week after transplantation. Rats were trained to undergo blood pressure measurements for 2 wk before the first measurement. A multichannel computerized system was used with tail cuffs and photoelectric sensors to detect the tail pulse (CODA; Kent Scientific, Torrington, CT). Rats were placed in restrainers while the temperature of the tail was maintained at 35–37°C. 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 with free access to water but not food (Bioquant; Merck, Darmstadt, Germany) for the collection of 24-h urine samples. Fasting blood samples were taken before transplantation and at the end of the study. Urinary and serum concentrations of total protein, creatinine, urea, sodium, and potassium were analyzed by a multitest system (Beckman-Coulter, Fullerton, CA) in the isograft control groups and survived until the end of the experiment. (χ^2 = 6.437, P = 0.09).

RESULTS

Survival. Renal allografts were transplanted in the DA-to-WF combination and isografts in the DA-to-DA combination. Allograft and isograft recipients were treated daily with spironolactone or vehicle until the end of the experiment at 12 wk after transplantation. During follow-up, three rats had to be killed prematurely (on days 40, 49, and 51 after transplantation) in the vehicle-treated allograft group and one rat (on day 51) in the spironolactone-treated allograft group (Fig. 1) due to a declined condition, as indicated by piloerected fur and severe weight loss (5–10% weight loss within 2 days). All rats in the isograft control groups survived until the end of the experiment (12 wk) (Fig. 1). Graft survival in spironolactone-treated allograft recipients tended to be improved (vs. vehicle-treated allograft recipients), without, however, reaching the level of statistical significance. The prematurely killed rats were ex-
cluded from all histological and biochemical analyses described below unless explicitly stated. Accordingly, for the long-term follow-up (12 wk), the following groups were studied: DA-to-WF allografts treated with vehicle \((n = 6)\), DA-to-WF allografts treated with spironolactone \((n = 8)\), DA-to-DA isografts treated with vehicle \((n = 8)\), and DA-to-DA isografts treated with spironolactone \((n = 8)\).

**Body weight.** Spironolactone treatment had no major effects on body weight of the recipients, although at 12 wk after transplantation a slightly decreased body weight was observed in spironolactone-treated allograft recipients compared with vehicle-treated allograft recipients (Fig. 2, solid gray line and solid black line, respectively). DA recipients had a significant lower body weight than WF recipients \((P < 0.05)\) throughout the experiment, independent of the treatment regimen.

**Blood pressure, urinary protein excretion, creatinine clearance, urea, and electrolytes.** Systolic blood pressure gradually increased in the allograft recipients without being affected by spironolactone treatment (Fig. 3A). Change in urinary protein excretion (expressed as the % change compared with levels before transplantation) in vehicle-treated allograft recipients increased significantly \((P < 0.05)\) during the experiment (from 6–12 wk after transplantation) compared with levels in isograft recipients (both vehicle- and spironolactone-treated) (Fig. 3B, solid black line). Spironolactone treatment in allograft recipients clearly reduced the increase in urinary protein excretion in time, without, however, reaching the level of statistical significance (Fig. 3B, solid gray line). The difference in absolute urinary protein excretion (expressed as mg/day) between vehicle- and spironolactone-treated allograft recipients at 12 wk also did not reach the level of statistical significance (Table 1). At 4 wk after transplantation, urinary volume (excreted in 24 h) was 19.4 ± 2.4 and 29.6 ± 2.8 ml \((\text{means} \pm \text{SE})\, P < 0.05\) in vehicle- and spironolactone-treated allograft recipients, respectively. Serum urea at 4 wk was 14.9 ± 1.5 and 22.5 ± 1.1 mmol/l \((\text{means} \pm \text{SE})\, P < 0.05\) in vehicle- and spironolactone-treated allograft recipients, respectively. Measurements of urinary volume, creatinine clearance, serum urea, electrolytes, and donor kidney weight before transplantation and at death 12 wk after transplantation are summarized in Table 1. In allograft recipients, spironolactone treatment increased serum urea \((P < 0.05)\) and urinary volume \((P = 0.05)\) and showed a trend toward increased urinary sodium excretion compared with these variables following vehicle treatment. In addition, spironolactone treatment slightly reduced the increase in kidney allograft weight. Spironolactone did not affect creatinine clearance, serum sodium, urinary potassium, or serum potassium.

Spironolactone treatment had no effect on any of the measured variables in isograft recipients.

**Development of TV.** Allografts presented with severe neoin-tima formation, indicative of TV, whereas in isografts, TV was hardly detected. Figure 4, A and B, shows photomicrographs of arteries with a diameter of ≥100 μm without and with TV, respectively. In allografts, mainly the larger intrarenal arteries with a minimal luminal diameter of ≥100 μm developed TV (Fig. 4C), whereas the smaller arteries were less prone to develop TV. Spironolactone treatment in allograft recipients...
significantly ($P < 0.05$) reduced the number of arteries with a diameter of $\geq 100 \mu m$ that were affected by TV, whereas no effects were observed in the smaller arteries (Fig. 4C). The total TV index, expressed as the average percent occlusion of all arteries $\geq 100 \mu m$ (including arteries with and without TV) pooled per group, was significantly decreased after spironolactone treatment (Fig. 4D). However, when only the arteries with TV are taken into account, the percent occlusion was not altered by spironolactone (Fig. 4E). Thus spironolactone reduces the number of arteries with a diameter $100 \mu m$ affected by TV, but not the severity of TV per se in affected vessels.

Histological examination of the four allografts retrieved from the prematurely killed rats revealed that all arteries with a diameter of $\geq 100 \mu m$ were affected by TV. The three vehicle-treated allografts had a total TV index of $47 \pm 9$ vs. $25 \pm 8\%$ in the long-term survivors of that group. The spironolactone-treated allograft had total TV index of $29\%$ vs. $7\%$ in the long-term survivors of that group.

Development of FGS and IF. PAS staining revealed that FGS was abundantly present in the allografts from vehicle-treated recipients, which was reduced by spironolactone, without, however, reaching the level of statistical significance (Fig. 5, A–C). Isografts remained free of FGS until the end of the experiment.

The amount of IF was significantly increased in both allografts retrieved from vehicle- and spironolactone-treated recipients compared with isografts. However, spironolactone did not ameliorate the development of IF in allografts (Fig. 5, D–F). Similar results for FGS and IF were obtained after analysis of Masson trichrome-stained sections (data not shown).

Histological examination of the four allografts retrieved from the prematurely killed recipients that these rats had severe IF. The three grafts retrieved from vehicle-treated recipients had an IF score of $167 \pm 21$ vs. $83 \pm 17\%$ in the long-term survivors of that group ($P < 0.05$). The allograft of the prematurely killed rat that was treated with spironolactone had an IF score of $133$ vs. $99 \pm 17\%$ in the long-term survivors of that group.

Glomerular and interstitial macrophage influx. Glomeruli of allografts from vehicle-treated recipients were characterized by marked infiltration with ED1$^+$ macrophages (Fig. 5G). Spiro- nolactone treatment significantly ($P < 0.05$) reduced the number of glomerulus-infiltrating macrophages (Fig. 5, H and I). Unlike glomeruli, spironolactone did not affect infiltration of macrophages in the interstitium (data not shown).

DISCUSSION

This study demonstrates that MR blockade by spironolactone significantly ameliorates TV in renal CTD in rats. Spi- ronolactone ameliorated the overall development of TV by reducing the number of arteries affected by TV, without, however, affecting the severity of TV in affected arteries. In addition, spironolactone treatment showed a trend toward reduced proteinuria and FGS, and a significant reduction of glomerular macrophage influx.

Spironolactone ameliorated the development of TV in renal allografts. At the moment, no effective therapies have been described that ameliorate TV following renal transplantation. TV is a specific lesion of chronic rejection (7, 35, 44), which leads to impaired perfusion and subsequent allograft dysfunction. Development of TV in CTD has been associated with reduced graft survival (9, 31, 41, 42, 49). We recently showed that also after experimental renal transplantation in rats, development of severe TV is associated with reduced survival (37). Also, in the present study, we found development of severe TV in the rats that had to be prematurely killed because of graft failure. These results support the hypothesis that spironolactone is a valuable therapy to ameliorate TV in CTD, thereby contributing to prolonged graft survival. Although spironolactone treatment of allograft recipients tended to improve graft survival, this did not reach the level of statistical significance most likely because of limited follow-up time (12 wk). Furthermore, other detrimental remodeling processes like development of interstitial fibrosis, which is not influenced by spironolactone in our model, contribute to graft loss. Amelioration of solely TV following spironolactone treatment may therefore not be sufficient to reach significant survival benefit at 12 wk after transplantation.

Spironolactone ameliorated TV by reducing the number of affected arteries rather than reducing the degree of occlusion. This suggests that MR activation by aldosterone plays a role in the initiation rather than the progression of neointima formation. Endothelial activation and damage play an important role in the initiation of TV (40). MR activation by aldosterone induces endothelial dysfunction (12, 19, 32), which can be ameliorated by MR blockade (11, 27). Proposed pathways for endothelial dysfunction are MR-induced upregulation of the epidermal growth factor receptor (24) and increased expression of ICAM-1, leading to leukocyte adhesion (4). Spironolactone

Table 1. Clinical variables from recipients before (pre-Tx) and at 12 wk after transplantation

<table>
<thead>
<tr>
<th></th>
<th>DA (pre-Tx) (n = 18)</th>
<th>Vehicle (n = 8)</th>
<th>Spironolactone (n = 8)</th>
<th>WF (pre-Tx) (n = 24)</th>
<th>Vehicle (n = 6)</th>
<th>Spironolactone (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urinary protein, mg/day</td>
<td>5.4±0.4</td>
<td>5.9±0.4</td>
<td>7.4±0.4</td>
<td>9.2±0.5</td>
<td>76.5±28.1</td>
<td>41.1±10.1</td>
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<tr>
<td>Urinary volume, ml</td>
<td>18±2</td>
<td>16±2</td>
<td>15±2</td>
<td>14±1</td>
<td>19±2</td>
<td>27±3†</td>
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<tr>
<td>Cr. clearance, ml/min</td>
<td>2.7±0.1</td>
<td>1.7±0.2</td>
<td>1.4±0.1</td>
<td>2.5±0.4</td>
<td>1.0±0.1</td>
<td>0.7±0.1</td>
</tr>
<tr>
<td>Serum urea, mmol/l</td>
<td>7.1±0.3</td>
<td>9.4±0.8</td>
<td>10.0±1.2</td>
<td>8.6±0.4</td>
<td>18.4±2.1</td>
<td>31.9±4.5*</td>
</tr>
<tr>
<td>Urinary sodium, mmol/day</td>
<td>0.54±0.02</td>
<td>0.35±0.05</td>
<td>0.53±0.05</td>
<td>0.75±0.03</td>
<td>0.85±0.18</td>
<td>1.01±0.15</td>
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<tr>
<td>Serum sodium, mmol/l</td>
<td>142±4.3</td>
<td>141±0.4</td>
<td>141±0.7</td>
<td>142.8±0.2</td>
<td>143±1.2</td>
<td>143±0.4</td>
</tr>
<tr>
<td>Urinary potassium, mmol/l</td>
<td>0.77±0.03</td>
<td>0.82±0.04</td>
<td>0.77±0.06</td>
<td>1.25±0.05</td>
<td>1.43±0.13</td>
<td>1.45±0.08</td>
</tr>
<tr>
<td>Serum potassium, mmol/l</td>
<td>5.8±0.1</td>
<td>4.4±0.1</td>
<td>4.4±0.1</td>
<td>6.0±0.05</td>
<td>4.7±0.3</td>
<td>4.8±0.1</td>
</tr>
<tr>
<td>Kidney weight, g</td>
<td>0.7±0.0</td>
<td>1.5±0.1</td>
<td>1.6±0.1</td>
<td>0.7±0.0</td>
<td>2.0±0.1</td>
<td>1.7±0.1</td>
</tr>
</tbody>
</table>

Values are means ± SE. DA, Dark Agouti; WF, Wistar-Furth. Statistical analyses were performed using transplanted (Tx) animals at 12 wk. Kidney weight of the donor kidney (from female DA rat pre-Tx). Cr., creatinine. *P < 0.05, †P = 0.05 vs. vehicle-treated allograft recipient.
potentially prevents the initiation of TV development by reducing MR-induced endothelial dysfunction, but this presumption needs further validation.

MR blockade reduced neointima formation in other models for vascular injury (5, 47, 48). This indicates that aldosterone can directly play a role in neointima formation by activation of smooth muscle cells, which was confirmed in vitro (22). However, in our study, MR blockade did not ameliorate the degree of neointima formation, suggesting that in this rat model for CTD other factors than aldosterone (for example angiotensin II) drive the progression of neointima formation.

In our study, proteinuria and FGS were slightly reduced in the spironolactone-treated allograft recipients. This is in line with observations in proteinuric models of native kidney disease where varying degrees of antiproteinuric efficacy of spironolactone are reported (6, 16, 17, 23, 38, 45). The increased

Fig. 4. Spironolactone ameliorated transplant vasculopathy (TV) by reducing the number of affected arteries. Spironolactone did not reduce the degree of neointima formation. A and B: photomicrograph of an artery from a vehicle-treated allograft without (A) and with TV (B). C: percentage of affected arteries present within small (25–49 μm), medium (50–99 μm), and large (≥100 μm)-diameter arteries. D: total TV index, expressed as the mean occlusion in all elastin-positive arteries with a diameter of ≥100 μm. E: percent occlusion in TV-affected arteries with a diameter of ≥100 μm. NI, neointima; arrowhead indicates elastin. Values (obtained at 12 wk) are means ± SE. *P < 0.05 vs. vehicle-treated allografts.
urinary volume and increased serum urea levels early in the course of treatment support a diuretic effect of spironolactone. Diuretic effects can contribute to reduction of proteinuria, probably by their effect on blood pressure. However, in our study, we did not observe an effect on blood pressure, suggesting that the modest effects on proteinuria and FGS cannot be solely explained by the diuretic effects of spironolactone. In our study, the amelioration of FGS and proteinuria coincided with reduction of glomerular macrophage influx, suggesting that the latter might be a mechanism for induction of FGS and proteinuria in this model (21). Aldosterone can upregulate ICAM-1, a recruitment factor of macrophages (46). The exact mechanism of aldosterone in the development of FGS and proteinuria in renal CTD and the amelioration thereof by spironolactone remains to be studied.

Remarkably, spironolactone did not ameliorate IF and the influx of interstitial macrophages in CTD, despite a reduction of TV and modest reduction of FGS and proteinuria. In a prior study in adriamycin-induced proteinuria, spironolactone had no effect on IF either (23). Although aldosterone has been shown to activate collagen production in rat renal fibroblasts (30), indicating a role of aldosterone in extracellular matrix accumulation in IF, other factors might be involved in the development of IF in vivo. In adriamycin-induced proteinuria, the combination of spironolactone and the angiotensin-converting enzyme inhibitor (ACEi) lisinopril did reduce IF, suggesting a crucial role for angiotensin II in the development of IF in the latter model. This might also apply to IF in CTD, which is supported by the observation that angiotensin II receptor blockade ameliorated development of IF in the Fischer 344-to-Lewis renal transplantation model for CTD (2). Our results do not allow a positive identification of the main mediators of IF in the current model of CTD, but apparently aldosterone is not the sole factor.

We administered spironolactone pretransplantation, to donors as well as recipients, and continued treatment in the recipients throughout the study. Consequently, our treatment regimen may have affected several consecutive triggers for...
CTD over time, such as ischemia-reperfusion injury (IRI), cyclosporine A (CsA) nephrotoxicity, and alloimmune rejection. MR activation has been associated with IRI and CsA nephrotoxicity (13, 28, 29, 33). In the present study, both allo- and isografts were exposed to IRI and all recipients received CsA treatment. As opposed to allografts, the isografts developed only subtle arterial and glomerular lesions, indicating that IRI and CsA are not the main triggers of development of these lesions. However, it is conceivable that IRI and CsA contribute to the effect of alloimmune rejection in the development of these lesions in the allografts. It is a limitation of our study that it does not allow for dissection between the different possible therapeutic effects of spironolactone that may have contributed to the eventual effect on CTD. Nevertheless, the effects on TV suggest that protection against the initiation rather than progression of arterial lesions is involved. This indicates that the early initiation of treatment with spironolactone (before transplantation) in our study has been relevant to the observed renoprotective effects. Nevertheless, based on the current results, we cannot exclude that besides the early initiation, the continued treatment contributed to the eventual outcome as well. It might also be that a combination of effects at different time points is important to obtain the therapeutic benefit of MR blockade on CTD. Further studies, with a design that allows a better resolution over time, are needed to determine the specific targets for MR blockade intervention in CTD and TV.

We are the first to show the effects of aldosterone MR blockade in allogeneic solid organ transplantation. MR blockade in animal models for native kidney diseases has been presented as a very potent mechanism to ameliorate renal dysfunction (6, 14, 16, 17, 34, 38, 45). In our rat model for CTD after allogeneic renal transplantation, MR blockade had prominent effects on intrarenal arterial lesions.

In conclusion, this study demonstrates that MR blockade by spironolactone ameliorates TV and reduces glomerular lesions to some extent in CTD in rats. Currently, many renal grafts are lost due to CTD and no effective treatment is available to ameliorate CTD. MR blockade has little side effects compared with standard immunosuppressive agents that are currently prescribed to renal transplant recipients. The current data therefore provide a rationale for exploration of the renoprotective potential of aldosterone blockade in the transplant setting.

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