Angiotensin II provokes podocyte injury in murine model of HIV-associated nephropathy

Hiroshi Ideura, Keiju Hiromura, Noriyuki Hiramatsu, Tetsuya Shigehara, Shigeru Takeuchi, Mai Tomioka, Toru Sakairi, Shin Yamashita, Akito Maeshima, Yoriaki Kaneko, Takashi Kuroiwa, Jeffrey B. Kopp, and Yoshihisa Nojima

1Department of Medicine and Clinical Science, Gunma University Graduate School of Medicine, Maebashi, Gunma, Japan; and 2Kidney Disease Section, Kidney Diseases Branch, National Institutes of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland

Submitted 7 April 2007; accepted in final form 17 July 2007

Ideura H, Hiromura K, Hiramatsu N, Shigehara T, Takeuchi S, Tomioka M, Sakairi T, Yamashita S, Maeshima A, Kaneko Y, Kuroiwa T, Kopp JB, Nojima Y. Angiotensin II provokes podocyte injury in murine model of HIV-associated nephropathy. Am J Physiol Renal Physiol 293: F1214–F1221, 2007. First published July 25, 2007; doi:10.1152/ajprenal.00162.2007.—Conditional transgenic mice that express one of the human immunodeficiency virus (HIV)-1 accessory genes, vpr, selectively in podocytes using a podocin promoter and a tetracycline-inducible system develop renal injuries similar to those of patients with HIV-associated nephropathy (HIVAN). We have shown that a heminephrectomy accelerates podocyte injury, which is alleviated by angiotensin II (ANG II) type 1 receptor blocker (ARB). The current study further explores the role of ANG II in the genesis of HIVAN in this murine model. With ANG II infusion, heavy proteinuria was observed at 1 wk after the initiation of doxycycline administration to induce vpr expression in podocytes. Severe morphological and phenotypical changes in the podocytes were observed at 2 wk, together with extensive glomerulosclerosis. Norepinephrine infusion, instead of ANG II, increased the systemic blood pressure to the same level as that achieved using ANG II. However, albuminuria and glomerular injury were modest in norepinephrine-infused mice. Treatment with an ARB, olmesartan, almost completely inhibited glomerular injury. In contrast, lowering the blood pressure with a vasodilator, hydralazine, partially decreased the exacerbation of podocyte injury (16). In the current study, to rule out the exacerbation of podocyte injury, we continuously infused ANG II in podocin/Vpr mice for simplicity) bearing a podocin/rtTA (recombinase system) construct in which vpr, nef, tat, rev, vif, vpu, and rev, only vpr and nef have been shown to result in the development of glomerular injury when expressed in podocytes alone (16, 37). Moreover, vpr and nef have a significant synergistic effect on podocyte injury. Although HIV-1 genes in podocytes are considered responsible for HIVAN, the mechanism responsible for podocyte damage leading to collapsing focal segmental glomerulosclerosis (FSGS) as a result of HIV-1 gene expression is not well understood.

Recently, our group (16) demonstrated that nephron reduction as a result of a heminephrectomy markedly accelerated vpr-induced podocyte injury and subsequent glomerular damage. We used dual transgenic mice (subsequently referred to as podocin/Vpr mice for simplicity) bearing a podocin/rtTA (reverse tetracycline transactivator) construct and a tetracycline-on (tetO)-vpr construct in which vpr expression is selectively induced in podocytes after the administration of doxycycline. We also demonstrated that angiotensin II (ANG II) type 1 receptor (AT1R) blockade almost completely inhibited the development and progression of renal injury in heminephrectomized-podocin/Vpr mice, suggesting that ANG II is involved in the exacerbation of podocyte injury (16). In the current study, to further examine the role of ANG II in vpr-induced podocyte injury, we continuously infused ANG II in podocin/Vpr mice instead of performing a heminephrectomy.

HUMAN IMMUNODEFICIENCY VIRUS (HIV)-associated nephropathy (HIVAN) is characterized by heavy proteinuria, rapidly progressive renal insufficiency, and distinct morphological changes in the kidney (2, 11, 20, 21, 28). The key histological features of HIVAN are segmental or global collapse of the glomerular capillary tuft, together with podocyte hypertrophy and hyperplasia as well as tubulointerstitial changes (2, 11, 20, 21, 28). Although the pathogenesis of HIVAN is not fully understood, the presence of the virus within renal cells, rather than immune dysregulation as a result of systemic HIV-1 infection, is considered to be important in the development of its characteristic histological changes (6, 23).

In human HIVAN, HIV-1 mRNA and DNA have been demonstrated in podocytes, parietal epithelial cells, renal tubular cells, and interstitial leukocytes (7, 32). In recently described transgenic mice, the selective expression of HIV-1 genes in podocytes was sufficient to evoke both glomerular and tubulointerstitial changes (16, 36, 37). Among the HIV-1 genes, including tat, rev, vif, vpu, vpr, and nef, only vpr and nef have been shown to result in the development of glomerular injury when expressed in podocytes alone (16, 37). Moreover, vpr and nef have a significant synergistic effect on podocyte injury. Although HIV-1 genes in podocytes are considered responsible for HIVAN, the mechanism responsible for podocyte damage leading to collapsing focal segmental glomerulosclerosis (FSGS) as a result of HIV-1 gene expression is not well understood.

MATERIALS AND METHODS

Animals. Podocin/rtTA transgenic mice (FVB/N background) and tetO/Vpr mice were generated as previously described (16). The mice were then crossbred to generate dual transgenic mice bearing both the podocin/rtTA and tetO/Vpr transgenes. Following the administration of doxycycline, vpr was selectively expressed in the podocytes of podocin/Vpr mice. Seven- or eight-week-old bitsagenic mice, weighing 20–25 g, were used for the experiments. The animals were maintained under specific pathogen-free conditions. Mouse husbandry

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
was carried out according to the protocols approved by the Animal Care Committee of Gunma University.

**Experimental protocol.** ANG II (1.44 mg·kg⁻¹·day⁻¹; Calbiochem, La Jolla, CA) was administered continuously using an osmotic pump (Alzet model 2004; Alza, Palo Alto, CA), which was implanted subcutaneously into the back of each podocin/Vpr mouse 2 days before the initiation of doxycycline administration. Doxycycline (Sigma-Aldrich, St. Louis, MO) was administered in drinking water (2 mg/ml) to induce vpr expression in the podocytes. Mice were also treated with olmesartan medoxomil (10 mg·kg⁻¹·day⁻¹; Sankyo, Tokyo, Japan) for 2 days before the experiment.

**Fig. 1.** Effect of continuous angiotensin II (ANG II) infusion in podocin/Vpr mice. Experimental protocol (A), urinary albumin excretion (B), and systemic blood pressure (C) in mice with ANG II infusion plus doxycycline (Dox) treatment (ANG II + Dox; n = 14), ANG II infusion alone (n = 8), or Dox alone (n = 8). A marked elevation of urinary albumin excretion was observed in ANG II-infused, Dox-treated mice. Increased systemic blood pressure was observed in ANG II-infused mice with or without Dox treatment.

*P < 0.01 vs. ANG II or Dox alone. **P < 0.01 vs. Dox alone; n.s., nonsignificant.

**Fig. 2.** Effect of continuous norepinephrine (NE) infusion in podocin/Vpr mice. Experimental protocol (A), urinary albumin excretion (B), and systemic blood pressure (C) in mice with NE infusion (n = 12) and ANG II infusion (n = 14). Both groups received Dox. Urinary albumin excretion was modest in NE-infused mice compared with ANG II-infused mice. No significant difference in increased systemic blood pressure was observed in either group. *P < 0.01 vs. ANG II.
To measure the systolic blood pressure in conscious mice, a UR-1000 device (Ueda Avancer, Tokyo, Japan) was utilized via an osmotic pump. Tail-cuff plethysmography performed using a Hitachi 7180 autoanalyzer (Hitachi High-Technologies, Tokyo, Japan).

Urinary and hematological analysis. Individual mice were placed in metabolic cages for 24-h urine collections. The urinary albumin concentration was determined using an ELISA kit (Albuwell M; Exocell, Philadelphia, PA). The serum albumin, total cholesterol, and urea nitrogen levels were assessed using a Hitachi 7180 autoanalyzer (Hitachi High-Technologies, Tokyo, Japan).

Histological analysis. Immunohistochemical staining was performed using paraffin-embedded kidney specimens as described previously. Briefly, 3-μm sections were deparaffinized, treated with hydrogen peroxide to block endogenous peroxidase, and microwaved to retrieve the antigens. The sections were then incubated overnight at 4°C with anti-Wilms tumor-1 (WT-1) antibody (Santa Cruz Biotechnology, Santa Cruz, CA), synaptopodin antibody (PROGEN, Heidelberg, Germany), or Ki-67 antibody (Lab Vision, Fremont, CA). After washing, the sections were incubated with a biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) followed by the Vectastain ABC reagent (Vector Laboratories) and diaminobenzidine (Nichirei, Tokyo, Japan). Slides were counterstained using methyl green or periodic acid-Schiff reagent (PAS).

For immunofluorescence analysis, the kidneys were quick-frozen in liquid nitrogen and stored at −80°C. Sections (3 μm) were fixed with cold methanol-acetone (1:1) for 10 min at −20°C. The sections were then incubated with anti-nephrin antibody (PROGEN) followed by incubation with FITC-conjugated anti-guinea pig IgG antibody (Molecular Probes, Eugene, OR).

Scoring of glomerulosclerosis and podocyte markers. Glomerular injury was evaluated by grading sclerosis and hyalinosis in the glomeruli on PAS-stained sections, using a score of 0 to 4 for each glomerulus. The percentage of area with sclerosis or hyalinosis was scored for each glomerulus as follows: 0, no lesion; 1, <25%; 2, 25 to <50%; 3, 50 to 75%; 4, >5% of the glomerular tuft, respectively. More than 50 sequential glomeruli from each mouse were evaluated, and the average of glomerulosclerosis and hyalinosis scores was calculated.

To evaluate the podocyte markers, the number of WT-1- and Ki-67-positive cells in each glomerulus was examined. For Ki-67 staining, the number of positive cells was counted among those cells located within Bowman’s space or on the glomerular basement membrane. Apparent parietal epithelial cells were excluded. More than 50 sequential glomeruli from each mouse were evaluated, and the average number of positive cells was calculated. For the evaluation of synaptopodin and nephrin expression, a semiquantitative grading system was used: 0, no stain; 1, weak staining; 2, intermediate staining; and 3, strong staining. The average staining score was calculated by counting more than 50 sequential glomeruli.

Statistical analyses. Data are means ± SD. Differences between experimental groups were evaluated using an ANOVA or Mann-Whitney test. Statistical significance was set at \( P < 0.05 \).

RESULTS

Continuous infusion of ANG II in podocin/Vpr mice. Podocin/Vpr mice express vpr, one of the HIV-1 accessory genes, selectively in podocytes after the administration of doxycycline. At 2–3 mo after the initiation of doxycycline administration, these mice exhibited renal injuries similar to those observed in patients with HIVAN (16). To determine the effect of ANG II in this mouse model of HIVAN, we continuously infused ANG II using a subcutaneously implanted osmotic pump (Fig. 1A). Podocin/Vpr mice showed trivial albuminuria (0.2 ± 0.1 mg/day) before the initiation of ANG II infusion.
After the administration of both ANG II and doxycycline, massive albuminuria was observed on day 9 (34.0 ± 19.5 mg/day), further increasing on day 16 (73.1 ± 43.3 mg/day) (Fig. 1B). In contrast, podocin/Vpr mice treated with ANG II alone or doxycycline alone showed a slight increase in albuminuria on day 16 (1.8 ± 0.9 and 2.6 ± 1.9 mg/day, respectively) (Fig. 1B). On day 16, the systolic blood pressure was elevated in ANG II-treated mice both with or without doxycy-
In ANG II-treated mice, the systolic blood pressure was slightly increased on day 2 (151 ± 10.2 mmHg), just before the administration of doxycycline, and more elevated on day 9 (171 ± 5.8 mmHg).

Effect of norepinephrine. To determine the effect of increased systolic blood pressure on albuminuria in podocin/Vpr mice, we next continuously infused the mice with norepinephrine, instead of ANG II (Fig. 2A). In mice treated with norepinephrine and doxycycline, increased albuminuria was observed on day 16 (4.1 ± 4.0 mg/day), but the level was much lower than that in mice treated with ANG II and doxycycline (Fig. 2B). The systolic blood pressure was similar in both groups on day 16 (Fig. 2C).

Effect of antihypertensive drugs. We next determined the effect of antihypertensive drugs in ANG II-infused podocin/Vpr mice treated with doxycycline (Fig. 3A). We used olmesartan, an ARB, and hydralazine, an arteriolar vasodilator. Olmesartan almost completely inhibited albuminuria on day 16 (0.4 ± 0.2 mg/day). In contrast, the mice treated with hydralazine showed only minor changes in these podocyte markers. Olmesartan, but not hydralazine, inhibited these changes.

Serological data for each group of mice

**Table 1. Serological data for each group of mice**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Day 0</th>
<th>Dox + Ang II</th>
<th>Dox + Ang II + Olm</th>
<th>Dox + Ang II + Hyd</th>
<th>Ang II</th>
<th>Dox + NE</th>
<th>Dox</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin, g/dl</td>
<td>3.0±0.1</td>
<td>1.9±0.3*</td>
<td>2.5±0.3</td>
<td>1.5±0.5*</td>
<td>3.0±0.3</td>
<td>3.0±0.6</td>
<td>2.8±0.3</td>
</tr>
<tr>
<td>T. chol, mg/dl</td>
<td>101±11</td>
<td>619±292*</td>
<td>138±33</td>
<td>504±180*</td>
<td>163±22</td>
<td>249±185</td>
<td>145±15</td>
</tr>
<tr>
<td>BUN, mg/dl</td>
<td>18.7±1.1</td>
<td>75.8±23.9*</td>
<td>31.1±8.7</td>
<td>60.2±27.5*</td>
<td>81.0±17.6*</td>
<td>42.8±20.2</td>
<td>27.1±5.9</td>
</tr>
</tbody>
</table>

Data are means ± SD. ANG II, angiotensin II; Dox, doxycycline; Olm, olmesartan; Hyd, hydralazine; NE, norepinephrine; T. chol, total cholesterol; BUN, blood urea nitrogen. *P < 0.05 vs. day 0.
Fig. 6. Immunostaining of podocyte markers and a proliferation marker. Renal sections from mice on day 0 and ANG II-infused, Doxy-treated mice on day 16 of treatment with or without Olm were immunohistochemically stained for Wilms tumor-1 (WT-1; A–C), synaptopodin (D–F), and Ki-67 (J–L). Sections were also stained using immunofluorescence for nephrin (G–I). ANG II-infused mice at day 16 showed decreased expression of WT-1, synaptopodin, and nephrin and increased expression of Ki-67 compared with the levels in mice on day 0. In contrast, Olm inhibited these changes. For Ki-67 staining, the number of positive cells was counted among those cells located within Bowman’s space or on the glomerular basement membrane (K, arrows). Slides were counterstained with methyl green (A–F) or PAS (J–L). Magnification: ×400.
to induce severe morphological changes in the glomerulus, although it induces mild proteinuria (18, 26). In the Habu venom toxin-induced nephritis, a model of mesangio proliferative glomerulonephritis, ANG II infusion markedly accelerates tubulointerstitial nephritis, together with heavy proteinuria and glomerulosclerosis (14). In the anti-Thy 1.1 nephritis, the other model of mesangio proliferative glomerulonephritis, the effect of ANG II is controversial. Continuous subcutaneous ANG II infusion ameliorates the early phase of mesangio proliferative glomerulonephritis (31, 34), whereas local delivery of ANG II using a type 1 collagen sponge exaggerates proteinuria in a rat progressive glomerulonephritis model, in which anti-Thy-1 antibody is injected, followed by a heminephrectomy (25). In the current study, we used podocin/Vpr mice, in which the expression of an HIV-1 gene, vpr, is induced selectively in podocytes after doxycycline administration, to examine the effect of continuous ANG II infusion on podocyte injury. We demonstrated that ANG II infusion strikingly accelerates proteinuria and glomerulosclerosis in the setting of vpr expression in podocytes.

ANG II-infused podocin/Vpr mice showed accelerated morphological and phenotypic changes in their podocytes. The extensive loss of the podocyte marker WT-1 and its maturity markers, synaptopodin and nephrin, was observed, together with an increased expression of Ki-67, a marker for cell cycle activation. Thus ANG II infusion enhanced the dysregulation and dedifferentiation of podocytes, leading to proteinuria and glomerulosclerosis. To our knowledge, this is the first report to demonstrate that ANG II infusion enhances obvious morphological and phenotypical changes in podocytes. How might ANG II affect podocyte injury? The adverse effects of ANG II on the progression of renal injury are thought to be associated with systemic and glomerular capillary hypertension and the profibrotic effects of ANG II (4). Our data show that the effect of ANG II on vpr-induced podocyte damage was independent of systemic blood pressure. Treatment with ARB, but not hydralazine, almost completely inhibited these changes. Norepinephrine infusion, instead of ANG II, also increased the systemic blood pressure to the same level as that induced by ANG II. However, norepinephrine did not induce heavy proteinuria or extensive histological changes.

We propose that two mechanisms may be involved in the acceleration of podocyte injury by ANG II. First, an increased intraglomerular capillary pressure may affect the podocytes. ANG II is a potent vasoconstrictor and is known to increase systemic blood pressure. In the glomeruli, ANG II is considered to increase glomerular capillary pressure by narrowing the efferent arterioles more than the afferent arterioles (12, 35). Thus increased glomerular capillary pressure may enhance morphological and phenotypical changes in the podocytes and lead to heavy proteinuria and glomerulosclerosis. Second, ANG II itself may directly affect the podocytes. In cultured podocytes, ANG II is reported to affect membrane voltage and conductance properties (15), the expression of heparan sulfate proteoglycans (5), the production of α3 (IV) collagen (9), and the cytoskeleton redistribution, including the shedding of nephrin (13, 24). The infusion of ANG II in isolated rat kidneys causes an impairment of the glomerular barrier, leading to the enhanced filtration of larger molecules and increased protein excretion (22). Transgenic rats expressing ATIR in podocytes develop albuminuria and structural podocyte damage that progresses to FSGS (17). Since we infused ANG II, not only ATIR but also ANG II type 2 receptor (AT2R) seems to be involved. However, our results show that AT1R blockade almost completely suppressed ANG II-induced podocyte injury, so we think that the aggravation of podocyte injury by ANG II in our model is largely dependent on AT1R, and the role of AT2R should be minimal. For now, we do not know whether increased intraglomerular capillary pressure or direct stimulation of ANG II is involved in the acceleration of vpr-induced podocyte injury. The precise mechanism of injury needs to be determined in the future.

Clinically, HIVAN is an important cause of end-stage renal disease (ESRD) in patients of African descent (10, 29). In early studies conducted in the pre-AZT (zidovudine) era, the median time to dialysis was 3–4 mo (8, 10, 28). By introducing antiviral therapy, especially the use of highly active antiretroviral therapy (HAART), the progression to ESRD has slowed (1, 30). In addition to antiviral therapy, the beneficial effect of angiotensin-converting enzyme inhibitors (ACEi) on HIVAN progression also has been reported (19, 33). The use of ACEi has been associated with enhanced renal survival in retrospective studies of patients with HIVAN, suggesting that ANG II is also involved in the exacerbation of renal injury in patients with HIVAN. Our data support the usage of ACEi or ARB for the treatment of HIVAN, especially for protection against the development and progression of podocyte injury resulting from the increased activation of the renin-angiotensin system.

In conclusion, we have shown that continuous ANG II infusion dramatically accelerated podocyte injury in a mouse model of HIVAN. Podocytes showed severe morphological and phenotypical changes under selective vpr expression and ANG II infusion. The effect of ANG II was independent of systemic blood pressure and was almost completely inhibited by AT1R blockade. These data demonstrate that excessive ANG II is critically involved in the development and progression of vpr-induced podocyte injury and subsequent glomerular damage.
ACKNOWLEDGMENTS

Olmesartan medoxomil was kindly supplied by Sankyo (Tokyo, Japan). We thank Rumiko Koitabashi for help in the preparation of kidney biopsy sections.

GRANTS

This work was supported in part by a Grant-in Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to K. Hiromura) and a Grant-in Aid from Gunma University (to K. Hiromura).

REFERENCES