Fluvastatin prevents podocyte injury in a murine model of HIV-associated nephropathy

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Abstract

Background. Recent studies have reported that statins have renoprotective effects, independent from lowering plasma cholesterol. In this study, we examined whether statins were beneficial in a murine model of HIV-associated nephropathy (HIVAN).

Methods. We used conditional transgenic mice that express one of the HIV-1 accessory genes, vpr, selectively in podocytes using podocin promoter and the Tet-on system. These mice develop aggressive collapsing focal segmental glomerular sclerosis with massive proteinuria and deterioration of renal function within 4 weeks following heminephrectomy and doxycycline administration. Fluvastatin was administrated simultaneously with doxycycline, and the effect was compared with untreated controls after 4 weeks.

Results. Fluvastatin at 10 mg/kg/day significantly decreased urinary albumin excretion (87 versus 11 mg/day, P < 0.01) and glomerular sclerosis (2.4 versus 1.0, P < 0.01, assessed by semi-quantitative scoring: 0–4). Fluvastatin also decreased serum creatinine and total cholesterol, but these differences were not statistically significant (0.36 versus 0.32 mg/dl, P = 0.35; 492 versus 378 mg/dl, P = 0.11, respectively). Phenotypic changes in podocytes, as indicated by the downregulation of nephrin, Wilms’ tumour 1 and synaptopodin, along with upregulation of proliferating cell nuclear antigen, were attenuated by fluvastatin, suggesting its protective effects against podocyte injuries. In cultured podocytes, angiotensin II treatment decreased nephrin expression to 13% of basal levels, which was reversed to 58% by adding fluvastatin.

Conclusions. In conclusion, fluvastatin was effective in treating experimental HIVAN. The beneficial effect of this drug might be caused, in part, by preserving nephrin expression in podocytes against angiotensin II-mediated injury.

Keywords: fluvastatin; HIV-associated nephropathy; podocyte

Introduction

HIV-1-associated nephropathy (HIVAN) occurs in ~10% of AIDS patients [1], the third leading cause of end-stage renal disease (ESRD) in African Americans between the ages of 20 and 64, and the most common cause of ESRD in HIV-1 seropositive patients [2]. The disease is most commonly characterized by collapsing focal segmental glomerular sclerosis (FSGS) defined by segmental or global collapse of the glomerular tuft associated with epithelial cell hypertrophy and hyperplasia [2].

Several studies have suggested that HIV may directly infect resident renal cells and cause glomerular injury. It has been demonstrated that the presence of HIV-1 DNA and mRNA in renal glomerular and tubular epithelial cells in human HIVAN proposed the role of kidneys as a viral reservoir [3,4]. Studies using animal models of HIVAN have suggested that the presence of virus within renal cells rather than immune dysregulation causes nephropathy. Zhong et al. generated transgenic mice in which HIV-1 genes, including vif, vpr, nef, tat and rev, were expressed selectively in podocytes by nephrin promoter [5]. These transgenic mice developed collapsing glomerulopathy after 4 weeks of age.

Our group has recently established inducible transgenic mice using the podocin promoter and Tet-on system [6,7]. In these mice, one of the HIV-1 accessory genes, vpr, was induced selectively in podocytes by the administration of doxycycline. These mice developed aggressive collapsing FSGS similar to human HIVAN with massive proteinuria and deterioration of renal function within 4 weeks following heminephrectomy and doxycycline administration [7]. Glomerular injury in this model was associated with phenotypic changes in podocytes, as indicated immunohisto-logically by the downregulation of nephrin, Wilms’ tumour 1 (WT-1) and synaptopodin, and the upregulation of proliferating cell nuclear antigen (PCNA).

Three-hydroxy-3-methylgultaryl coenzyme A (HMG-CoA) reductase inhibitors, also known as statins, are extremely useful cholesterol-lowering agents. There is increasing evidence of the beneficial effects of statins.
unrelated to their lipid-lowering capacity, in preventing various disease conditions including myocardial infarctions, ischaemic strokes and rejection of transplanted hearts [8–10]. Recent in vivo studies have reported that statins also have renoprotective effects independent from lowering cholesterol. For example, treatments with statins were effective in the model of acute renal failure caused by ischaemia-reperfusion injury, and in cases of tubulointerstitial nephritis caused by chronic cyclosporine administration or unilateral ureteral obstruction [11–13]. The beneficial effects of statins in glomerular injury have also been demonstrated in many studies. Treatment with statins proved to be effective in anti-Thy1 glomerulonephritis, Heymann nephritis, streptozotocin-induced nephropathy and nephrotoxic serum-induced nephritis, which are murine models of mesangial proliferative nephritis, membranous nephropathy, diabetic nephropathy and crescentic glomerulonephritis, respectively [14–17].

More importantly, several studies have suggested that statins attenuate glomerular injuries through distinct podocyte-protective effects. Administration of fluvastatin decreased proteinuria and alleviated podocyte injury in puromycin aminonucleoside (PAN)-induced nephrosis [18]. Immunofluorescence studies demonstrated that fluvastatin treatment dramatically reversed nephrin and podocin reductions in this model. In the transgenic Ren2 rats, which harbour the mouse renin transgene, structural changes consistent with periarteriolar fibrosis and podocyte foot-process effacement were attenuated with statin treatment. Glomerular nephrin expression was also diminished in these mice and tended to normalize with statin treatment [19]. Collectively, these data suggest that statins may be effective in other glomerulopathies triggered by podocyte injury. However, the benefits of statins in a mouse model of HIVAN characterized by collapsing FSGS have yet to be reported.

In this experiment, we investigated the effects of fluvastatin in a mouse model of HIVAN using our recently established heminephrectomized bitransgenic podocin/Vpr mice. We found that fluvastatin treatment significantly decreased both urinary albumin excretion and glomerulosclerosis. The potential mechanism of efficacy by fluvastatin was examined by immunohistological and in vitro studies. Our results suggested that it was, in part, through direct podocyte-protective effects.

Subjects and methods

Antibodies and chemicals

Guinea pig polyclonal anti-nephrin antibodies to the extracellular fibronectin domain (GP-N2) and mouse monoclonal anti-synaptopodin antibodies (G1D4) were from Progen (Heidelberg, Germany). Rabbit polyclonal anti-WT-1 antibodies (C-19) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal PCNA-Ab-1 (PC10) antibodies were from NeoMarkers (Fremont, CA, USA). Alexa Fluor® 488-labeled anti-guinea pig antibodies were from Molecular Probes (Eugene, OR, USA). Angiotensin II was from Calbiochem (Darmstadt, Germany). Fluvastatin was obtained from Novartis Farma (Junebu, Switzerland).

Experimental mouse models of HIVAN

An experimental model of HIVAN, bitransgenic podocin/Vpr mice (FVB/N background) bearing both podocin-rtTA and tetO-vpr transgene was developed as previously described [6,7]. These mice express vpr selectively in podocytes by doxycycline administration, resulting in collapsing FSGS. We also found that, in these mice, heminephrectomy markedly enhanced proteinuria excretion and glomerulosclerosis, designated as accelerated podocin/Vpr mice. In the present experiment, we used accelerated podocin/Vpr mice as an animal model of HIVAN. Right heminephrectomy was performed in male mice at 8–12 weeks of age. After 1 week, doxycycline at a dose of 2 mg/ml in drinking water was orally administered to mice with or without fluvastatin at a dose of 1 or 10 mg/kg/day for 4 weeks. The number of mice used in this experiment is as follows: untreated (n = 9), fluvastatin-treated (1 or 10 mg/kg/day) HIVAN (Flu1; n = 8, Flu10; n = 8) mice at 4 weeks of doxycycline administration. Each value represents mean ± SD. *P < 0.05 versus untreated; **P < 0.05 versus Flu1.

Urine and serum analyses

Individual mice were placed in metabolic cages for 24-h urine collections at 4 weeks after the initiation of doxycycline administration. Urinary albumin concentration was determined by an ELISA kit (Albuwell M; Exocell, Philadelphia, PA, USA). Serum creatinine, urea nitrogen, total protein, albumin, total cholesterol and triglyceride levels were assessed by a Hitachi 7180 autoanalyser (Hitachi High-Technologies Corp., Tokyo, Japan).

Histological analysis

Paraffinized kidney tissue sections (3 µm thick) were deparaffinized, placed in 0.01 M of sodium citrate buffer (pH 6.0) and heated twice for 5 min in a microwave oven. After inactivation of endogenous peroxidase with 0.5% metaperiodic acid in phosphate-buffered saline (PBS) for 10 min, the sections were blocked for 10 min with a protein block (Dako Cytomation, Carpinteria, CA, USA). The sections were then incubated with anti–WT-1 antibodies (C-19) (1:200), anti-synaptopodin antibodies (G1D4) (1:1) or anti-PCNA-Ab-1 (PC10) antibodies (1:200) at 4°C overnight and subsequently with biotinylated anti-mouse or anti-rabbit IgG for secondary antibodies. Immunoreactivity was detected using an ABC kit (Vector Laboratories, Burlingame, CA, USA). After washing with PBS, the signals were finally developed with diaminobenzidine (Nichirei, Tokyo, Japan). Slides were counterstained using methyl green or periodic acid–Schiff reagent (PAS). For nephrin, frozen sections of the kidney tissues
(3 µm thick) were blocked for 10 min with a protein block and then incubated with polyclonal anti-nephrin antibodies (1:200) at 4°C overnight. After washing with PBS, the sections were incubated with Alexa Fluor® 488-labelled anti-guinea pig antibodies (1:1000) at room temperature for 1 h. After washing with PBS, the signals were observed using fluorescence microscopy.

**Scoring of glomerulosclerosis and podocyte markers**

Glomerular damage was evaluated by grading sclerosis in the glomeruli on PAS-stained sections, using a score of 0–4 for each glomerulus, as previously described [7,20]. Briefly, the percentage of area with sclerosis was scored for each glomerulus as follows: 0, no lesion; 1, <25%; 2, 25–<50%; 3, 50–<75%; 4, >75% of the glomerular tuft, and the average of glomerulosclerosis scores was calculated. To evaluate the podocyte markers, the number of WT-1- and PCNA-positive cells in each glomerulus was examined and the average number of positive cells was calculated. For the evaluation of synaptopodin and nephrin expression, a semi-quantitative grading system was used: 0, no; 1, weak; 2, intermediate; and 3, strong staining, and the average staining score was calculated. More than 50 sequential glomeruli from each mouse were evaluated.

**Cell culture**

The immortalized mouse podocyte cell line was kindly provided by Dr Stuart J. Shankland and cultured as previously described [21]. In brief, undifferentiated podocytes were cultured in RPMI-1640 (Sigma) medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 10 U/ml of interferon-γ supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal calf serum, 100 U/ml penicillin. The immortalized mouse podocyte cell line was kindly provided by Dr Stuart J. Shankland and cultured as previously described [21].

**Immunofluorescence staining of the mouse podocyte cell line**

Differentiated podocytes were seeded at 1 x 10^5 cells/ml in an eight-well Lab-Tek® Chamber Slide® system (Nunc, NY, USA), precoated with type I collagen (Sigma; C9191) and stimulated with 1 µM of angiotensin II with or without 3 µM of fluvastatin for 24 h, and then fixed with paraformaldehyde at 37°C for 30 min. After washing with PBS, the cells were incubated with a protein block for 10 min. The cells were then incubated with anti-nephrin antibodies (1:200) at 4°C overnight and subsequently with Alexa Fluor® 488-labelled anti-guinea pig antibodies (1:1000) and 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI) at room temperature for 1 h. After washing with PBS, the signals were observed using fluorescence microscopy. The expression of nephrin was evaluated by quantitative analysis of nephrin/DAPI immunofluorescence intensity using image J.

**Statistical analysis**

Data were expressed as means ± SD. Differences between experimental groups were evaluated by one-way ANOVA or by the Mann–Whitney test. P < 0.05 was regarded as statistically significant.

**Results**

**Fluvastatin decreased proteinuria and glomerulosclerosis in HIVAN**

We first examined the effect of fluvastatin treatment on proteinuria and glomerulosclerosis in HIVAN. As a model of HIVAN, heminephrectomized bitransgenic podocin/Vpr mice were employed. In these mice, podocyte-specific expression of the HIV-1 accessory gene, vpr, was induced by doxycycline administration. Its expression led to the development of collapsing FSGS, which was markedly enhanced by prior heminephrectomy. A week after heminephrectomy, doxycycline was orally administered to mice. Fluvastatin was also orally administered at 1 or 10 mg/kg/day simul-
Effect of fluvastatin on HIV AN

Table 1. Serological data of each mouse group

<table>
<thead>
<tr>
<th></th>
<th>WT (n = 5)</th>
<th>Untreated (n = 9)</th>
<th>Flu10 (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP (g/dl)</td>
<td>4.4 ± 0.4</td>
<td>4.3 ± 0.5</td>
<td>4.1 ± 0.3</td>
</tr>
<tr>
<td>Alb (g/dl)</td>
<td>2.9 ± 0.2</td>
<td>2.1 ± 0.2</td>
<td>2.2 ± 0.4</td>
</tr>
<tr>
<td>Cr (mg/dl)</td>
<td>0.12 ± 0.03</td>
<td>0.36 ± 0.20</td>
<td>0.32 ± 0.17</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>28.4 ± 4.9</td>
<td>138.3 ± 58.9</td>
<td>115.8 ± 60.3</td>
</tr>
<tr>
<td>T-cho (mg/dl)</td>
<td>127.6 ± 4.9</td>
<td>492.2 ± 179.0</td>
<td>377.5 ± 193.0</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>60.4 ± 25.4</td>
<td>226.0 ± 110.1</td>
<td>147.5 ± 75.3</td>
</tr>
</tbody>
</table>

WT, wild type; Flu10, fluvastatin at 10 mg/kg/day; TP, total protein; Alb, albumin; Cr, creatinine; BUN, blood urea nitrogen; T-cho, total cholesterol; TG, triglyceride.

Data are mean ± SD.

Fluvastatin preserved nephrin expression in HIVAN

Nephrin plays a critical role in maintaining the glomerular filtration barrier, thus regulating urinary protein excretion [22]. A previous study revealed that glomerular injury in this model is associated with a decrease of nephrin expression [7]. Accordingly, we hypothesized that fluvastatin may decrease proteinuria by modulating nephrin expression. To explore this, glomerular nephrin expression was examined by immunofluorescence staining. Its intensity was evaluated by semi-quantitative analysis (score: 0, no; 1, weak; 2, intermediate; and 3, strong staining). The kidney of wild-type mice was used as a control; its average score was 2.9. As shown in Figure 3, nephrin expression was decreased in untreated HIVAN mice; this was significantly reversed by fluvastatin treatment (0.5 ± 0.1 versus 2.2 ± 0.3, P < 0.01).

Fluvastatin improved phenotypic changes of podocytes in HIVAN

Glomerular injury in this model is initiated by phenotypic changes of podocytes, as indicated by the downregulation of WT-1 (a podocyte marker) and synaptopodin (a podocyte differentiation marker), and the upregulation of PCNA (a podocyte dedifferentiation marker). To investigate the podocyte-protective effects by fluvastatin, we semi-quantitatively analysed the glomerular expression of WT-1, synaptopodin and PCNA (Figure 4, Table 2). For WT-1 and PCNA, the positive cell number per glomeruli was counted. For synaptopodin, a score from 0 to 3 (score: 0, no; 1, weak; 2, intermediate and 3, strong staining) was used. In untreated HIVAN, the expressions of both WT-1 and synaptopodin were much lower than those of controls (P < 0.01, for both), while PCNA expression was higher (P < 0.01). As expected, fluvastatin treatment significantly reversed these changes. Compared with the untreated group, the expressions of WT-1 and synaptopodin were higher and that of PCNA was lower in the fluvastatin-treated group (P < 0.01, for all).
In HIVAN mice, fluvastatin treatment reversed the reduction of glomerular nephrin expression (Figure 3). This might be an important mechanism by which fluvastatin decreased proteinuria. We finally examined whether fluvastatin directly modulated nephrin expression in cultured podocytes. In this HIVAN model, our group previously identified that angiotensin II played a critical role in podocyte injury, because phenotypic changes of podocytes such as nephrin reduction were promoted by angiotensin II administration and completely inhibited by an angiotensin II type 1 receptor blocker [7,20]. Therefore, we chose angiotensin II as a stimulant to mimic podocyte injury in vitro. Immortalized mouse podocytes were grown and stimulated with angiotensin II in the presence or absence of fluvastatin. After 24-h incubation, cellular nephrin expression was assessed by semi-quantitative immunofluorescence staining, as described in the Subjects and methods section. As shown in Figure 5, angiotensin II treatment significantly decreased nephrin expression to 13% of basal level \((P < 0.01)\). This reduction was again reversed to 58% by adding fluvastatin \((P < 0.01)\).

**Discussion**

Accumulating evidence suggests that statins have renoprotective effects independent from reducing cholesterol synthesis. However, their efficacy in HIVAN is largely unknown. In this study, we demonstrated that fluvastatin significantly decreased proteinuria and glomerulosclerosis in the bitransgenic podocin/Vpr mice, a murine model of HIVAN. A dramatic decrease of proteinuria by this drug seems to result from a pleiotropic effect regardless of its cholesterol-lowering profile, because the differences in plasma cholesterol levels between fluvastatin-treated and untreated mice were not statistically significant.

In immunohistological analysis, fluvastatin treatment preserved nephrin expression, which was substantially decreased along with disease progression. *In vitro*, fluvastatin also preserved nephrin expression in cultured podocytes against angiotensin II stimulation. Although glomerular injuries in HIVAN mice were associated with phenotypic changes in podocytes, such as decreases of WT-1 and synaptopodin and an increase of PCNA, these changes were significantly reversed by fluvastatin, indicating its podocyte-protective effect. Taken together, these results suggest that fluvastatin is effective in treating HIVAN mice independent from its lipid-lowering profile. The beneficial effect of this drug might be caused, in part, by preserving nephrin expression in podocytes against angiotensin II-mediated injury.

Shibata et al. reported that fluvastatin treatment significantly decreased proteinuria and serum creatinine elevation in rats with PAN-induced nephrosis [18]. In histological analysis, nephrin expression showed diminished and discontinuous staining patterns, which was dramatically improved by fluvastatin. In renin-transgenic rats, podocyte foot-process effacement and diminished glomerular nephrin expression were improved by statin treatment [19]. Tonolo et al. demonstrated that in patients with microalbuminuric type II diabetes, simvastatin treatment preserved renal function and increased the mRNA expression of slit diaphragm proteins, including nephrin and podocin, in kidney biopsy specimens [23]. In a study that examined the efficacy of statins in patients with chronic glomerulonephritis, both proteinuria and urinary podocyte excretion were significantly decreased after 6 months of cerivastatin treatment [24]. Collectively, these data support the idea that statins have distinct podocyte-protective effects, especially on the preservation of nephrin expression.

We previously reported that severe proteinuria, glomerulosclerosis and phenotypic changes in podocytes were dramatically inhibited by an angiotensin II receptor blocker in HIVAN mice [7]. Of note, there is an interesting observation that in passive Heymann nephritis, statin treatment directly decreased renal angiotensin-converting enzyme (ACE) activity by 30% [15]. Considering the critical role of angiotensin II in HIVAN mice, the efficacy of statin might result from a decrease in local angiotensin II production through the inhibition of renal ACE activity. Given the importance of angiotensin II in renal damage, the next question is how angiotensin II causes glomerular injury. Such pathways can be the therapeutic targets of statins. In the current study, we showed that treatment with angiotensin II decreased nephrin expression in cultured podocytes and this reduction was partially reversed by fluvastatin. Macconi et al. also showed that angiotensin II decreased nephrin expression in cultured podocytes through the reorganization of F-actin fibres and redistribution of zona occludens-1 [25]. Collectively, these data support the idea that angiotensin II causes podocyte injury through a decrease of nephrin expression.

An important issue is whether the therapeutic dose of fluvastatin used in mice can translate into that used in humans. The maximal dose of fluvastatin approved for the treatment of hyperlipidaemia in human is 80 mg/day, approximately equivalent to 1 mg/kg/day. In our experiment, fluvastatin at 1 mg/kg significantly reduced proteinuria, but 10 mg/kg was required to achieve histological improvement.
Although pharmacological data obtained in rodents cannot be directly applied to humans, these data suggest that approved dosage of fluvastatin reduces proteinuria in human HIVAN, but higher dose is required to expect histological improvement. Similar dose-related effects of statins have been reported by Kourliouros et al. on atrial fibrillation after cardiac surgery [26]. The highest dose had the greatest preventative effect. An intermediate dose was also effective although to a lesser degree, while low dose did not influence postoperative atrial fibrillation.

In our experiment, fluvastatin was started with doxycycline administration, before glomerular injuries were developed. In this context, we should be careful to expand our findings to that fluvastatin exerts therapeutically beneficial effects after the development of HIVAN. Before translating fluvastatin into clinical usage, data need to be collected investigating its effect on the established disease to answer an important question as to whether it allows stabilizing or even reversing HIVAN.

In conclusion, fluvastatin was effective in treating an experimental model of HIVAN, independently from lipid-lowering profile. The beneficial effect of this drug might have resulted, in part, from preserving nephrin expression in podocytes against angiotensin II-mediated injury. Despite the fact that HIVAN is an important cause of ESRD in the United States, no prospective controlled trials evaluating the fact that HIVAN is an important cause of ESRD in the United States, no prospective controlled trials evaluating treatment for HIVAN have been described. Only retrospective studies suggest the efficacy of some currently available agents such as anti-retrovirals, ACE inhibitors and corticosteroids [27–29]. Statins are safe and widely used agents, and our data provide support for the clinical use of statins in patients with HIVAN.

Acknowledgements. We thank Dr Stuart J. Shankland for providing the immortalized mouse podocyte cell line. We also thank Ms Rumiko Koitabashi for her technical assistance. This work is supported in part by a grant from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

Conflict of interest statement. None declared.

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Received for publication: 8.9.08; Accepted in revised form: 6.1.09