Effect of atorvastatin on dendritic cells of tubulointerstitium in diabetic rats

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Inflammatory reactology has become increasingly important in diabetic kidney disease. In this study, we established STZ-induced diabetic rat model to investigate whether dendritic cells (DCs) mediated tubulointerstitial damages, and whether the effects by DCs were mediated by P-selectin expression and can be inhibited by atorvastatin. The study demonstrated that there was an accumulation of DCs in diabetic rats mediated by P-selectin. It also showed the accumulation of DCs and expression of P-selectin was closely correlated with the degree of renal tubulointerstitial injury. These effects were markedly attenuated by atorvastatin. Thus, DCs play a role in tubulointerstitial damages, atorvastatin can prevent renal tubulointerstitial from damage by inhibiting the P-selectin expression and DCs migration. [BMB reports 2010; 43(3): 188-192]

INTRODUCTION

Diabetic kidney disease (DKD) is one of the most common and severe complications of diabetes mellitus. Although previous investigation in diabetes has focused on the glomerulus, tubulointerstitial injury has become a major feature of DKD (1). Some investigations indicate that the tubulointerstitial damages in DKD take place even earlier than glomerulus, and that the severity of tubulointerstitial damages is closely correlated to renal hypofunction and affects prognosis directly (2). Inflammatory reactology had been recently accepted by many researchers, indicating that infiltrated inflammatory cells and immune reaction are responsible for those tubulointerstitial damages (3). The inflammatory cells include lymphocytes, mononuclear macrophages and dendritic cells, the latter of which has been proved to be mediated by P-selectin (4). Until recently, there are few reports on the relationships between DCs and tubulointerstitial diseases in DKD. Given the fact that lymphocytes and mononuclear macrophages could evoke inflammatory factors of renal tubulointerstitial lesions in DKD and atorvastatin can exert renal protective effect by anti-inflammation (1, 5), in our current study, we intent to investigate the relationship between DCs and tubulointerstitial damages and the role of atorvastatin on the accumulation of DCs in DKD by establishing STZ-induced diabetic rats model.

RESULTS

General indexes
As shown in Table 1, blood lipid, Scr and urinary Alb/Cr ratio were increased in diabetic rats. Treatment with atorvastatin did not ameliorate lipid abnormalities but mitigated Scr and urinary Alb/Cr (Table 1).

Pathological changes
In diabetic rats, the scores at week 4, 8, and 12 were 1, 2 and 3, respectively. Treatment with atorvastatin attenuated the damages in tubularinterstitium compared with untreated diabetic rats in the same period.

Renal expression of P-selectin protein
While P-selectin protein expression was rarely observed in the kidney of control rats, it was increased in diabetic rats among renal tubulointerstitium, especially on tubular epithelial cells. At week 4 after hyperglycemia, the expression of P-selectin protein was weak but dramatically increased at week 8, and reached the peak at week 12. The expression of P-selectin protein was significantly decreased with atorvastatin treatment, compared with the untreated diabetic rats in the same period (Fig. 1, 2).

Renal distribution of DCs
CD1a+CD80+ DCs (4) were mainly distributed in tubulointerstitium in the early stage of DKD, rarely appeared in normal renal tissues or abnormal glomerulus. The distribution was prominently greater in diabetic rats at week 12 than that of at week 4 or at week 8. Treatment with atorvastatin reduced the accumulation of DCs, compared with the untreated rats in the same period (Fig. 3).
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Table 1. Changes of triglyceride (TG, mmol/l), total cholesterol (TC, mmol/l), Scr (μ mol/l), Alb/cr (g/mol) in different groups at different periods

<table>
<thead>
<tr>
<th>Group</th>
<th>4 W</th>
<th>8 W</th>
<th>12 W</th>
<th>4 W</th>
<th>8 W</th>
<th>12 W</th>
<th>4 W</th>
<th>8 W</th>
<th>12 W</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG</td>
<td>0.85±0.13</td>
<td>0.91±0.15</td>
<td>0.87±0.18</td>
<td>2.34±0.12</td>
<td>2.29±0.36</td>
<td>2.38±0.29</td>
<td>2.23±0.31</td>
<td>2.19±0.41</td>
<td>2.18±0.37</td>
</tr>
<tr>
<td>TC</td>
<td>1.63±0.14</td>
<td>1.72±0.18</td>
<td>1.69±0.21</td>
<td>2.19±0.20</td>
<td>2.17±0.24</td>
<td>2.21±0.19</td>
<td>2.15±0.23</td>
<td>2.07±0.17</td>
<td>1.99±0.21</td>
</tr>
<tr>
<td>Scr</td>
<td>55.29±9.58</td>
<td>57.35±10.03</td>
<td>56.62±10.52</td>
<td>98.54±15.33</td>
<td>125.44±13.47</td>
<td>182.00±15.82</td>
<td>73.58±14.97</td>
<td>94.47±16.03</td>
<td>106.51±15.47</td>
</tr>
<tr>
<td>Alb/Cr</td>
<td>1.75±0.18</td>
<td>1.69±0.21</td>
<td>1.74±0.23</td>
<td>111.45±21.77</td>
<td>194.52±23.88</td>
<td>415.05±38.53</td>
<td>63.89±14.07</td>
<td>103.90±18.02</td>
<td>260.23±25.08</td>
</tr>
</tbody>
</table>

*p < 0.01, vs N group; ^p < 0.01. *p < 0.05, **p > 0.01, vs D group; n=7.

**DISCUSSION**

It is believed that infiltrated inflammatory cells and inflammatory immune response in tubulointerstitium are the main reasons of tubulointerstitial damage, and the degree of renal tubulointerstitial damage is closely related to renal failure and prognosis. DCs are the most potent antigen presenting cells in the immune system; they are involved in the initiation of primary immune responses, autoimmune diseases, graft rejection, human immunodeficiency virus infection, and the triggering of adaptive immunity (6). Studies have also demonstrated that DCs present in animal and human renal tissue (7). In addition, some scholars have pointed out that DCs possess the highest proportion in interstitial immune cells and DCs presented a variety of pathological types of glomerulonephritis (8). Many researchers demonstrate that the immune regulatory function of DCs is related to its mature state of development. In the inflammatory condition, immature DCs can migrate from blood circulation to peripheral tissue under the mediation of adhesion molecule P-selectin and chemokines, capturing and processing antigen. After maturation, DCs generate antigen-presenting effect initiating the local inflammatory immune response (7, 9, 10). However, the regulation mechanism of migration and function of DCs in DKD renal tissue is still unclear.

In this study, DKD was induced by STZ. After induction, there were early symptoms on diabetic kidney damage, including increases in blood glucose, serum creatinine, urinary albumin/creatinine ratio and kidney volume. In addition, various

![Fig. 1](image1)

![Fig. 2](image2)
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Fig. 3. (1-7) The distribution of dendritic cells in rat renal tissue of different groups at different periods (dual label stain, multifunctional microscope ×400); (8) Numbers of dendritic cells analyzed by multifunctional microscope system were graphically shown. Lane 1: D group at week 4; Lane 2: T group at week 4; Lane 3: D group at week 8; Lane 4: T group at week 8; Lane 5: D group at week 12; Lane 6: T group at week 12. *P < 0.01, versus D group at the same period.

pathological changes occurred in tubulointerstitium, including tubulointerstitial thickening, light fibrosis and inflammatory cells infiltration. Compared with the control rats, expression of P-selectin was up-regulated among tubulointerstitial at week 4 in diabetic rats, followed by CD1a+CD80+DCs infiltration in tubular, renal interstitium and renal blood vessel, mainly in renal interstitium. At week 8, expression of P-selectin was markedly increased along with increased DCs infiltration in diabetic rats. Both the expression of P-selectin and DCs infiltration showed a peak at week 12 in diabetic rats, with tubulointerstitial injury appeared at the same time. There were a significantly strong correlation between the levels of CD1a+CD80+ DCs accumulation and P-selectin expression. All of these results indicate that DCs accumulation among renal tubulointerstitial mediated by P-selectin in diabetes was probably closely correlated with pathogenesis of DKD tubulointerstitial injury. Considering the present outcomes that we have and DC biological function (9, 11), we hypothesize two mechanisms by which DCs may mediate tubulointerstitial injury: Firstly, DCs are an important resource in several pro-inflammatory factors and pro-fibrotic factors including TNF-α, IL-6 and TGF-β, which lead tubulointerstitial inflammation and subsequent development of interstitial fibrosis; Secondly, DCs might migrate to local lymph nodes to present renal auto-antigens to T and B lymphocytes, directing renal autoimmune response.

Atorvastatin is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMA-CoA) reductases. Our laboratory has demonstrated that statin can reduce the expression of monocyte chemotactant protein (MCP-1) to prevent renal injury (1). In this study, several observations were obtained by comparing with diabetic rats at the same period: the expression of P-selectin was down-regulated, tubulointerstitial pathological changes were lessened, serum creatinine and urinary albumin/creatinine ratio were decreased, and accumulation and distribution of CD1a+CD80+ DCs in tubulointerstitium were markedly inhibited in the rats treated with atorvastatin. It also showed that there were no significant differences in plasma glucose and lipid levels between the rats of the D and T group. All of these results indicate that atorvastin had a renal protective effect on diabetic kidney disease. The effect of atorvastatin was partially due to its ability of reducing expression of P-selectin and inhibiting accumulation of DCs in tubulointerstitium (12). This kind of protective effect of atorvastatin might not dependent on blood glucose and lipid metabolism.

In conclusion, DCs infiltration among renal tubulointerstitium which were mediated by P-selectin could be closely correlated with the progression of tubulointerstitial fibrosis. Atorvastatin interfered the expression of adhesion molecules P-selectin by blocking the mediating role of DCs, thereby re-
ducing the participation of DCs in immune inflammatory re-
action in tissues. This may be one of the mechanisms where
atorvastatin reduces anti-inflammatory effect on kidney in dia-
betic rats.

MATERIALS AND METHODS

Experimental animals and reagents
Male rats were purchased from Animal Experiment Center of
Wuhan University. The reagents used in the experiments in-
cluded LIPITOR (atorvastatin calcium, Pfizer, USA), STZ (stre-
ptozotocin, Sigma, USA), anti-CD1a, anti-P-selectin antibody
and ECL reagent (Santa Cruz, USA), anti-CD80 antibody, P-se-
lectin immunohistochemistry SP kits (Bo Shi De, China).

Animal model, groups and correlation detection
Rats were received an intraperitoneal injection of STZ (60
mg/kg) dissolved in citric buffer. These rats that had a glucose
≥ 16.7 mmol/L after 72 h injection were considered to be dia-
betic rats. The diabetic rats were randomly separated into two
groups as the diabetic control group (D group, included twenty-
one rats) and the atorvastatin treatment group (T group, in-
cluded twenty-one rats). Those rats injected only with citric
acid buffer were used as the normal control group (N group,
included twenty-one rats). Those rats in the T group were ad-
ministered atorvastatin at a dose of 20 mg/kg/d. Seven rats of
each group were sacrificed at week 4, 8 and 12, respectively.

Perfusion fixation
Chest and abdominal cavity were exposed in anesthetized rats.
The ascending aorta was dissociated from the heart after with-
drawing blood. A small vantage was cut in left ventricular tip
and the perfusion needle was transmitted into the aorta. The
right atrial appendage was cut at the same time to run off the
blood. We harvested the renal tissue until the kidney turned
white and harder. The perfusate was 4% paraformaldehyde
and ECL reagent (Santa Cruz, USA), anti-CD1a, anti-P-selectin antibody
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white and harder. The perfusate was 4% paraformaldehyde
and triglycerides levels.

Pathological changes
Pathological changes were evaluated by HE staining and
Masson staining. Semi-quantitative scoring method was proc-
essed as following: 0 = normal interstitium and tubules; 1 =
light fibrosis, mild tubulointerstitial thickening, increased tubu-
lointerstitial cell infiltration; 2 = light to moderate fibrosis and
tubulointerstitial thickening, significant increase in infiltrating
cells; 3 = moderate fibrosis, significant interstitial thickening,
tubular expansion and significant increase in infiltrating cells.
A total of 20 visions from each biopsy slide was selected and
assessed.

Immunohistochemistry
Immunohistochemistry was performed to determine the ex-
pression of P-selectin protein in kidney. Three-micron-thick
sections were prepared after tissues were fixed and paraffin-
embedded. Following dewaxed and subjected to heat-mediated
antigen retrieval in a microwave, anti-P-selectin antibody
at ratio of 1 : 100 was added into these sections and in-
cubated overnight at 4°C. The next day, 1 : 100 anti-biotin an-
tibody was added into these sections and incubated for 30 min
at 37°C. Later, these colors of these sections were developed
by DAB, hematoxylin, dehydrated, cleared and mounted. PBS
was used as a negative control instead of the primary antibody.
Semi-quantitative analysis methods were used to analyze the
results: 40 tubulointerstitial visions were randomly selected
with HPIAS-1000 high-definition medical image analysis sys-
tem at 400 times fields. The depth and scope of the positive
staining were expressed as average absorbance (A).

Western blot
Western blot was performed to determine the expression of
P-selectin protein in kidney. Equal amounts of proteins (50
μg/lane) were separated by 8% SDS-PAGE and electro-blotted
onto a nitrocellulose membrane. The membrane was stained
with Ponceau S to verify the blotting result. The membrane
was blocked for non-specific binding in nonfat milk (5% in
TBST) for 2 h at 4°C and then incubated with 1 : 200 anti-P-se-
lectin antibody overnight. After further incubation with 1 :
1,000 anti-rabbit IgG for 1 h at room temperature, the mem-
brane was developed using ECL reagent and exposed to film.
Images were scanned and the densitometric analysis was per-
formed on a computer.

Dual-label immunofluorescence staining assay
Distribution of DCs in renal tissue was assayed by dual-label
immunofluorescence staining (4, 14, 15). Three-micron-thick
sections were conventional dewaxing, repaired by microwave,
incubated with anti-CD1a antibody (working concentration
1 : 100) and anti-CD80 antibody (working concentration
1 : 100) overnight at 4°C. Subsequently, the sections were
added with anti-goat IgG-FITC and anti-rabbit IgG-TRITC
(workng concentration 1 : 200), respectively. The sections
were incubated for 2 h at 37°C, washed with PBS and then
mounted. The primary antibody was replaced by PBS to be a
negative control. The sections were observed and images were
captured by Olympus micro-camera system. CD1a was pos-
tively stained by green-fluorescence, and CD80 was positively
stained by red-fluorescence. Cells with dual staining as shown
by yellow-fluorescence represented DCs (CD1a+CD80+). DCs
count in kidney tissue: 20 high power microscope fields of vi-
sions (400 times) in each section were randomly selected, and
the number and density of DC yellow-fluorescence were
measured. All the data about measurement results were trans-
ferred into Excel data files for further analysis.

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Statistical analysis
SPSS software version 13.0 was used for statistical analysis. Observation indexes were all expressed as $\chi \pm s$. One-way ANOVA was used for multi-group comparisons, while the t-test was used for comparisons between two groups. Significant difference are defined as $P < 0.05$.

REFERENCES