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Original Article

HMG-CoA reductase inhibitor ameliorates diabetic nephropathy by its pleiotropic effects in rats

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Abstract

Background. An inflammatory process may be one of the critical factors that contribute to the development of diabetic nephropathy (DN). We reported previously that intercellular adhesion molecule-1 (ICAM-1) is up-regulated and promotes macrophage infiltration in the glomeruli of diabetic rats. 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) have recently been emphasized to have anti-inflammatory effects; inhibition of leukocyte adhesion and migration, independent of the cholesterol-lowering effect. The present study was designed to test the hypothesis that statins prevent the development of DN by pleiotropic effects.

Methods. Streptozotocin-induced diabetic rats were treated with cerivastatin (0.5 mg/kg body weight) or vehicle for 4 weeks. We analysed glomerular macrophage infiltration and ICAM-1 expression. We also evaluated major regulators of ICAM-1, activation of nuclear factor-kappa B (NF-κB) using electrophoretic mobility shift assay, and oxidative stress.

Results. Statin treatment reduced urinary albumin excretion (UAE) (2.96 ± 0.18 *vs* 2.38 ± 0.06; $\log_{10}$ UAE, $P < 0.05$), glomerular size ($12\,150 \pm 329$ *vs* 9963 ± 307 μm^2, $P < 0.05$), and lowered blood pressure, compared with untreated diabetic rats. Immunohistochemistry revealed that macrophage infiltration and ICAM-1 expression in glomeruli were increased in diabetic rats and were inhibited by statin treatment. Renal NF-κB activity, urinary excretion and renal deposition of 8-OHdG were increased in diabetic rats, and reduced by statin treatment.

Conclusion. Statin treatment prevented glomerular injury, independent of the cholesterol-lowering effects. Our findings suggest that the beneficial effect might be mediated by pleiotropic effects including an anti-inflammatory action through a reduction of oxidative stress, NF-κB activation, ICAM-1 expression and macrophage infiltration in the early phase of DN.

Keywords: diabetic nephropathy; HMG-CoA reductase inhibitor; ICAM-1; macrophage; NF-κB; oxidative stress

Introduction

Diabetic nephropathy (DN) is one of the major causes of end-stage renal failure worldwide. Although angiotensin converting enzyme inhibitors (ACEI) and angiotensin II receptor antagonists are effective in DN, inhibition of the renin–angiotensin system may not be sufficient to completely prevent disease progression. Clarification of the pathogenesis of DN and development of novel and effective therapeutic strategies are therefore high priorities.

The postulated molecular mechanisms in the progression of DN include accumulation of advanced glycation end-products (AGEs), activation of protein kinase C (PKC), acceleration of the polyol pathway and over-expression of transforming growth factor-β. In addition to these factors, the emerging roles of inflammatory processes, such as over-expression of cell adhesion molecules and chemokines, which induce leukocyte infiltration, are recognized in DN. Besides the accumulation of extracellular matrix, infiltration of mononuclear cells is another pathogenic feature in the glomeruli of patients with DN. We showed previously that over-expression of leukocyte adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1) and infiltration of macrophages both in the glomeruli and interstitium of patients with DN [1]. We further demonstrated that up-regulated expression of ICAM-1 on glomerular endothelial cells plays a critical role in the recruitment of macrophages into

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glomeruli of streptozotocin (STZ)-induced diabetic rats in such an early stage as 1–2 weeks [2]. Expression of ICAM-1 can be induced by multiple factors, including inflammatory cytokines, reactive oxygen species and shear stress [2,3]. In addition, nuclear factor-kappa B (NF-κB) is one of the most important transcription factors regulating ICAM-1 expression [4].

Recently, accumulating evidence has suggested that 3-hydroxy-3-methylglutanyl coenzyme A (HMG-CoA) reductase inhibitors (statins) have anti-inflammatory and endothelial cell protective actions that are independent of the cholesterol-lowering effect [5]. Statins may influence intracellular pathways that are involved in the inflammatory and fibrogenic responses in progressive renal injury [5]. A recent report by Park *et al.* [6] suggested that statins ameliorate renal injury by reduction of macrophage infiltration, expression of adhesion molecules, and activation of nuclear transcription factors including NF-κB and activating protein-1 (AP-1), independent of blood pressure- and cholesterol-lowering effects. In the experimental animal model of DN, several studies have revealed the effects of statins on DN [7]. Although some hypotheses were postulated and discussed in previous reports, the precise mechanisms of beneficial effects of statins have still remained unclear.

The present study was designed to investigate whether statins could prevent the development of DN through reduction of ICAM-1 expression and macrophage infiltration, independent of the cholesterol-lowering effect. Moreover, we evaluated NF-κB activation and oxidative stress, as strong regulators of ICAM-1.

Subjects and methods

Experimental protocol

Experiments were conducted in three groups of 5-week-old male Sprague–Dawley (SD) rats (Charles River Japan, Yokohama, Japan) with initial body weights of 116 ± 9.3 g. The rats had free access to standard chow and tap water. All procedures were performed according to the Guidelines for Animal Experiments at Okayama University Medical School, Japanese Government Animal Protection and Management Law (No. 105) and Japanese Government Notification on Feeding and Safekeeping of Animals (No. 6). We divided the rats into three groups: (i) non-diabetic control rats (Non-DM); (ii) STZ-induced diabetic rats that received vehicle alone (DM) and (iii) diabetic rats that were treated with cerivastatin (0.5 mg/kg body weight $\cdot$ day^{-1}, DM + statin). Diabetic groups were injected with 65 mg/kg body weight of STZ (Sigma Chemical Co., St Louis, MO) intraperitoneally at the age of 5 weeks. We measured their blood glucose 1, 2, 3, 7, 14 and 28 days after the STZ injection, and included only rats with a blood glucose concentration >16 mmol/l in the study. The DM + statin group received cerivastatin for 4 weeks once daily, starting the day prior to the STZ injection. The Non-DM and DM groups received vehicle alone. After 4 weeks, rats were killed and both kidneys were dissected and weighed. The final number of animals of each group was five. For the NF-κB electrophoretic mobility shift assay (EMSA), kidney tissues were snap frozen in liquid nitrogen and stored at -80°C. Cerivastatin sodium was provided by Bayer Yakuhin, Ltd (Osaka, Japan).

Metabolic data

At 4 weeks, systolic blood pressure was measured by the tail-cuff method. Blood samples were collected at 2 and 4 weeks. Haemoglobin A1c (HbA1c) was measured by the high-pressure liquid chromatography (HPLC) method, and serum cholesterol was measured by cholesterol oxidase-HDAOS method. Serum creatinine was measured using the enzymatic method. Urine samples were collected over a 24 h period in individual metabolic cages at 2 and 4 weeks. The urinary albumin concentration in a 24 h urine collection was measured by nephelometry using anti-rat albumin antibody (ICN Pharmaceuticals, Aurora, OH). Data of urinary albumin excretion (UAE) was logarithmically transformed.

Light microscopy

Renal tissues were fixed in 10% formalin and embedded in paraffin. Paraffin sections (4 μm) were then stained with periodic acid-methenamine (PAM). To evaluate glomerular size, 50 glomeruli per animal were examined. Glomerular surface area was measured by manually tracing the Bowman's capsule and using Photoshop software version 6 (Adobe systems, San Jose, CA) and NIH image analysis software version 1.62. The results were expressed as mean $\pm$ SEM μm^2.

Immunoperoxidase staining for macrophages

Infiltration of macrophages was evaluated by immunoperoxidase staining using the ABC kit (Vector Laboratories, Burlingame, CA) as described previously by our group [2]. Briefly, ethanol-fixed sections were cut at 4 μm thickness. To reduce the background, non-specific binding was blocked by incubating with 10% normal goat serum in Tris-buffered saline for 20 min. Non-specific staining was blocked by 15 min incubation, with avidin and then biotin using the avidin–biotin blocking kit (Vector Laboratories). Endogenous peroxidase activity was inhibited by 20 min incubation with methanol containing 0.3% H_2O_2. Sections were first incubated with a monoclonal antibody (mAb) against rat monocytes/macrophages (ED1) for 12 h at 4°C. Sections were then incubated with biotin-labelled goat anti-mouse IgG for 30 min. Biotinylated horseradish peroxidase was applied for 30 min. Peroxidase activity was developed in 3,3-diaminobendine (DAB). Mayer's haematoxylin was added as a counter stain.

Intraglomerular ED1-positive cells were counted in 50 glomeruli per animal under high power field by two independent observers with no prior knowledge of the experimental design. The average number per glomerulus was used for the estimation.

Immunofluorescence staining for ICAM-1

ICAM-1 expression was detected by indirect immunofluorescence as described previously [2]. Briefly, fresh frozen sections (4 μm) were stained with a mAb against rat ICAM-1 (1A29) for 12 h at 4°C. They were then stained with FITC-conjugated rabbit-anti-mouse IgG for 30 min at room temperature. Sections were observed by a confocal laser fluorescence microscope (LSM-510, Carl Zeiss, Jena, Germany).

Quantification of ICAM-1 immunofluorescence intensity was calculated by the modified method as described previously by Toyokuni *et al.* [8]. Briefly, colour images were obtained as PICT files by LSM-510. The brightness of each image file was uniformly enhanced by Photoshop, followed by analysis using NIH image software. PICT image files were inverted and opened in grey scale mode. The ICAM-1 index was calculated using the formula, {[X (density) × positive area (μm^2)] / glomerular total area (μm^2)}, where the staining density is indicated by a number from 0 to 256 in grey scale.

Electrophoretic mobility shift assay

Tissue extracts and EMSA for the transcription factor NF-κB were performed as described earlier [9]. Briefly, frozen kidney cortex was minced and suspended in 1 ml of TBS buffer [25 mM Tris–HCl (pH 7.4), 130 mM NaCl and 5 mM KCl] and homogenized with a tube-homogenizer s-303 (Ikeda Scientific Co., Tokyo). The homogenates were centrifuged and the pellets re-suspended in 1 ml of buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT)] and chilled on ice for 20 min. Next, 100 µl of 10% Nonidet P-40 was added and vigorously vortexed. The nuclear fraction was collected by centrifugation and resuspended in 100 µl of buffer B [20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 1 mM PMSF]. For EMSA, 50 µg of nuclear extract was incubated with 1 ng of [γ-^{32}P]ATP-labelled oligonucleotide (ODN), containing an NF-κB binding site (5′-AGTTGAGGGGACTTCCCAGGC-3′) in 20 µg binding buffer [10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 1 mM $MgCl_2$, 4% glycerol and 50 mg/ml dI–dC] for 30 min at room temperature. Individual samples were then electrophoresed on a 4% polyacrylamide gel. The gel was dried and exposed to X-ray film (Hyperfilm-MP; Amersham Pharmacia Biotech Inc., Piscataway, NJ). The relative intensity of the autoradiogram was determined by a scanning densitometer. The result of densitometry scanning was presented as mean ± SEM ($n = 5$ for each groups). To assess the specificity of the reaction, we performed a competition assay with 50-fold excess of unlabeled consensus sequences of NF-κB. The unlabelled probes were added to the binding reaction 10 min prior to the labelled probe. Each experiment was performed three times.

Urinary 8-OHdG excretion

As a marker for oxidative DNA damage, we measured the urinary excretion of 8-OHdG. The urinary 8-OHdG concentration in a 24 h urine collection was measured by enzyme-linked immunosorbent assay using the 8-OHdG ELISA Kit (Japan Institute for the Control of Aging, Shizuoka, Japan) [8].

Immunoperoxidase staining for 8-OHdG

To detect the localization of 8-OHdG, immunoperoxidase staining was performed using the ABC kit as described previously [8]. In brief, ethanol-fixed sections were prepared, and antigen retrieval was performed using a microwave. Antibodies against 8-OHdG were used for the primary reactions followed by secondary reactions with biotin-labelled anti-mouse goat IgG. Other steps were performed as described above.

Quantification of immunoperoxidase staining for 8-OHdG in glomeruli was calculated by modified method as described previously by Toyokuni *et al.* [8]. The glomerular 8OH-dG index was calculated using the formula, {[X – threshold (density) × positive area (μm^2)] / glomerular total area (μm^2)}. Staining density is indicated by a number from 0 to 256 in grey scale of NIH image. Threshold was set as 50, since the density of nuclear counter staining was less than 50. Fifty glomeruli per animal were evaluated.

Antibodies

The primary antibodies used were anti-rat ICAM-1 mAb (1A29) (Seikagaku Corporation, Tokyo, Japan), anti-rat monocyte/macrophage mAb (ED1) (Serotec, Oxford, UK) and anti-8-OHdG mAb (NOF Corporation, Tokyo, Japan). The secondary antibodies were biotin-labelled goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA) and FITC-conjugated rabbit-anti-mouse IgG (Jackson Immunoresearch Laboratories).

Statistical analysis

Data are presented as means ± SEM. Differences between groups were examined for statistical significance using one-way analysis of variance (ANOVA) followed by Scheffe analysis. A P-value < 0.05 was considered a statistically significant difference.

Results

Metabolic data

Body weights of both DM and DM + statin groups were lower than those for the non-DM group. Systolic blood pressure increased in the DM group. The DM + statin group revealed lower blood pressure compared with the DM group without statistical significance. HbA1c, total cholesterol levels and creatinine clearance were elevated in DM and DM + statin groups. However, there was no significant difference between them (Table 1). UAE was higher in the DM group. Statin treatment significantly reduced UAE compared with the DM group (Table 1). Kidney weights of both DM and DM + statin groups were increased compared with non-DM group. Statin treatment reduced the mean kidney weight compared with the DM group (Table 1).

Kidney morphology

The glomerular surface area was increased in the DM group ($12\,150 \pm 329\ \mu m^2$) compared with the non-DM group ($9988 \pm 363\ \mu m^2$, $P < 0.05$). Statin treatment significantly reduced the glomerular surface area ($9963 \pm 307\ \mu m^2$) compared with the DM group ($P < 0.05$) (Figure 1).

Macrophage infiltration and ICAM-1 expression in glomeruli

The number of macrophages (ED1-positive cells) in glomeruli was increased in the DM group compared

Table 1. Changes in metabolic data after induction of diabetes

Parameters	Non-DM	DM	DM + statin
Body weight (g)			
Week 0	112 ± 3.8	117 ± 0.9	112 ± 1.2
Week 2	224 ± 9.6	189 ± 6.5[a]	184 ± 8.8[a]
Week 4	316 ± 6.9	229 ± 13.7[a]	197 ± 17.8[a]
Blood pressure (mmHg) (week 4)	99.6 ± 6.3	125.5 ± 3.9	100.8 ± 10.9
HbA1c (%)			
week 0	1.32 ± 0.09	1.36 ± 0.03	1.40 ± 0.06
week 4	2.6 ± 0.2	6.5 ± 0.2[c]	6.6 ± 0.3[c]
T chol (mmol/l)			
week 0	2.35 ± 0.19	2.22 ± 0.49	1.89 ± 0.05
week 4	1.11 ± 0.13	1.83 ± 0.21[a]	2.01 ± 0.17[a]
S-Cr (μmol/l)			
week 0	22.1 ± 0.53	23.8 ± 1.94	23.8 ± 3.09
week 2	28.3 ± 1.77	23.8 ± 1.50	23.8 ± 1.51
week 4	28.3 ± 1.77	29.2 ± 2.65	24.8 ± 1.59
Ccr ($ml.s^{-1}100\ gBW^{-1}$)			
week 0	12.8 ± 1.72	12.2 ± 1.67	12.9 ± 2.31
week 2	14.2 ± 0.98	25.6 ± 2.40[a]	29.6 ± 2.31[a]
week 4	13.5 ± 1.1	16.8 ± 1.44	18.9 ± 1.22
Log_{10} (UAE(μg/24h))			
week 0	1.98 ± 0.10	2.2 ± 0.25	2.35 ± 0.13
week 2	2.22 ± 0.05	2.48 ± 0.06	2.47 ± 0.09
week 4	2.27 ± 0.03	2.96 ± 0.18[a]	2.38 ± 0.06[b]
Kidney weight (g/100gBW) (week 4)	401.6 ± 5.4	668.9 ± 12.6[c]	628.3 ± 18.2[c]

Data are mean ± SEM. [a]$P < 0.05$ *vs* Non-DM, [b]$P < 0.05$ *vs* DM and [c]$P < 0.0001$ *vs* Non-DM.
T chol, total cholesterol; S-Cr, serum creatinine; Ccr, creatinine clearance.

with the non-DM group. Statin treatment significantly reduced glomerular macrophage infiltration. The number of ED1-positive cells per glomerulus was as follows: non-DM *vs* DM *vs* DM + statin: 0.9 ± 0.35 *vs* 2.2 ± 0.10 *vs* 1.4 ± 0.08, Figures 2A–C and 3).

ICAM-1 expression was increased on glomerular endothelial cells (Figure 2D–F) and peritubular capillaries in the interstitium (Figure 2G–I) in the DM group, but was markedly reduced in the DM + statin group compared with the DM group (Figure 3).

NF-κB activation

Using EMSA, we analysed the activation of NF-κB DNA binding activity in the kidney, which is one of the regulators of ICAM-1. The result of densitometry scanning for NF-κB activation was shown as the relative intensity to lane 4 (background level). NF-κB activity was increased in the kidneys of the DM group (relative intensity 69.6 ± 35.89) compared with the non-DM group (13.5 ± 1.91, $P < 0.05$). Statin treatment reduced renal DNA binding activity of NF-κB (12.7 ± 4.92) compared with the DM group ($P < 0.05$) (Figure 4).

Oxidative stress

Using 8-OHdG, we analysed oxidative stress as a factor stimulating NF-κB activation and ICAM-1 expression. Urinary excretion of 8-OHdG was markedly higher in the DM group than in non-DM (non-DM *vs* DM *vs* DM + statin: 241.5 ± 15.3 *vs* 389.9 ± 25.4 *vs* 282.3 ± 38.9 ng/day, Figure 5A). Statin treatment reduced urinary 8-OHdG excretion to control levels. Immunoperoxidase staining of 8-OHdG revealed over-expression of 8-OHdG in glomeruli and tubuli in diabetic kidneys. Statin treatment also reduced renal 8-OHdG expression (Figure 5C–E). Glomerular 8-OHdG index was significantly elevated in the DM group and normalized by statin therapy (Figure 5B).

Discussion

In the present study, statin treatment of STZ-induced diabetic rats resulted in amelioration of albuminuria and glomerular hypertrophy, without altering serum cholesterol levels. Our findings suggest that these reno-protective effects are independent of cholesterol-lowering effect.

Statin treatment reduced both the expression of ICAM-1 and the infiltration of macrophages in diabetic glomeruli. Macrophages are considered to contribute to the progression of DN through secretion of various cytokines and nitric oxide. Depletion of leukocytes by irradiation improves glomerular dysfunction in the early phase of DN in STZ-induced diabetic rats [10]. As we described previously, ICAM-1 plays a pivotal role in infiltration of macrophages in the early phase of DN. Neutralizing antibody against ICAM-1

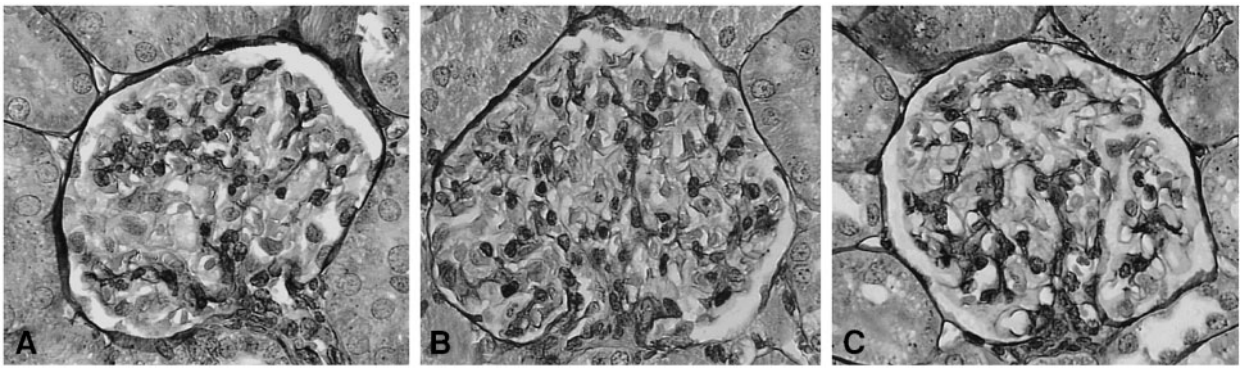

Fig. 1. Representative glomeruli from non-diabetic controls (**A**), untreated diabetics (**B**) and statin-treated rats (**C**). Glomerular hypertrophy was evident in the untreated diabetic group, but glomerular size was reduced by statin treatment. (PAM staining, ×400.)

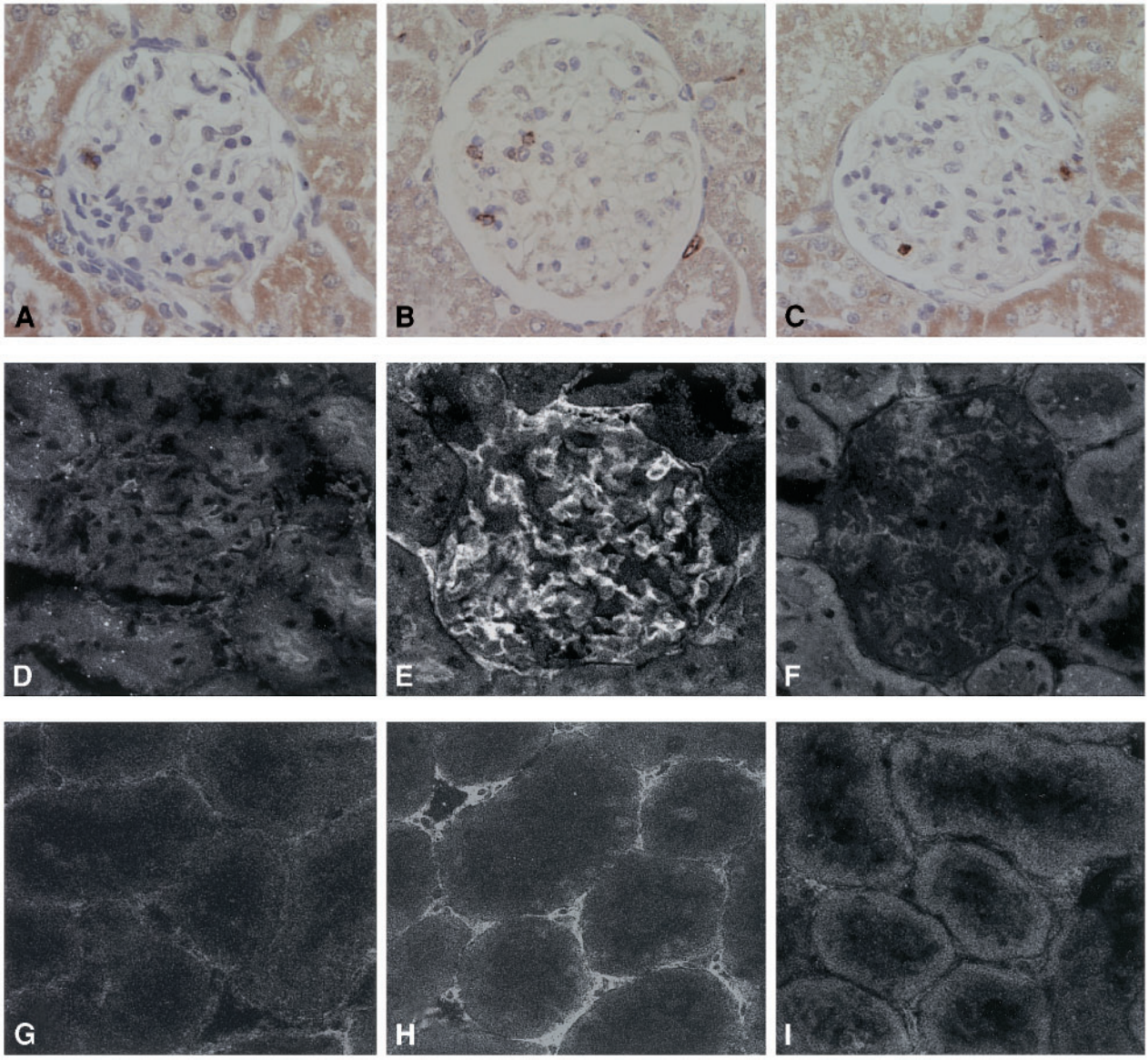

Fig. 2. Immunoperoxidase staining for macrophages (ED1-positive cells) in glomeruli (**A**–**C**). Immunofluorescence staining for ICAM-1 in glomeruli (**D**–**F**) and peritubular capillaries (**G**–**I**). Kidney tissues were taken from non-diabetic controls (A, D and G), untreated diabetics (B, E and H) and statin-treated rats (C, F and I) (×400).

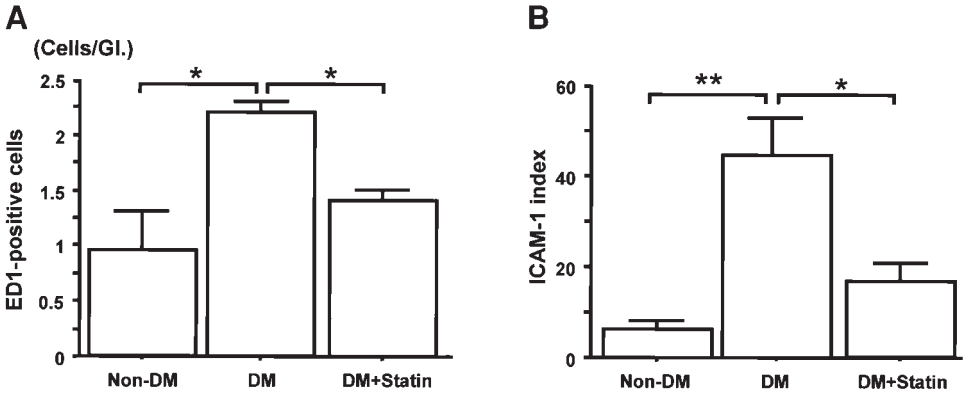

Fig. 3. Number of macrophages (ED1-positive cells) (**A**) and immunofluorescence intensity for ICAM-1 (ICAM-1 index) (**B**) in glomeruli of non-diabetic controls (Non-DM) untreated diabetics (DM) and statin-treated rats (DM + statin). Values are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.001$.

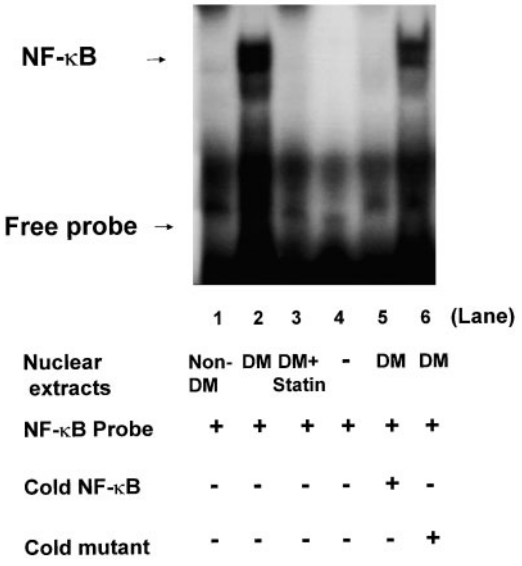

Fig. 4. Electrophoretic mobility shift assay for NF-κB in the kidneys. Lanes 1–4, NF-κB activation. Lane 1, non-diabetic controls (non-DM); lane 2, untreated diabetics (DM); lane 3, statin-treated rats (DM + statin); lane 4, without nuclear extract; lanes 5 and 6, specificity of NF-κB DNA binding; lane 5, pretreated with excess cold NF-κB; lane 6, pre-treated with excess cold mutant NF-κB. (Individual samples were electrophoresed. $n = 5$ for each group. Only one of each group were presented as figure.)

blocked macrophage infiltration into diabetic glomeruli [2]. Furthermore, Sadeghi *et al.* [11] demonstrated that statins inhibit ICAM-1 expression in endothelial cells *in vitro*.

In line with these notions, it is suggested that cerivastatin prevented glomerular damage in early stage of DN via inhibition of ICAM-1-dependent infiltration of macrophages into glomeruli in rats. The inflammatory changes may also be involved in fibrotic and sclerotic processes in later phase. This process is not investigated in this study and remains to be solved.

We can speculate several possible mechanisms for the reduction of ICAM-1 expression and macrophage infiltration: (i) inactivation of NF-κB, (ii) reduction of oxidative stress, (iii) inactivation of RhoA and (iv) inhibition of lymphocyte function associated antigen-1 (LFA-1), which is one of the major counter receptors for ICAM-1.

Statin treatment reduced activation of the transcription factor NF-κB in this study. NF-κB is one of the major factors involved in ICAM-1 transcription [4]. In diabetic state, many factors such as AGEs, shear stress and oxidative stress [4] contribute to NF-κB activation. Hofmann *et al.* [12] indicated that insufficient glycemic control increases NF-κB activation in peripheral blood mononuclear cells isolated from patients with type 1 diabetes. Other studies also showed that

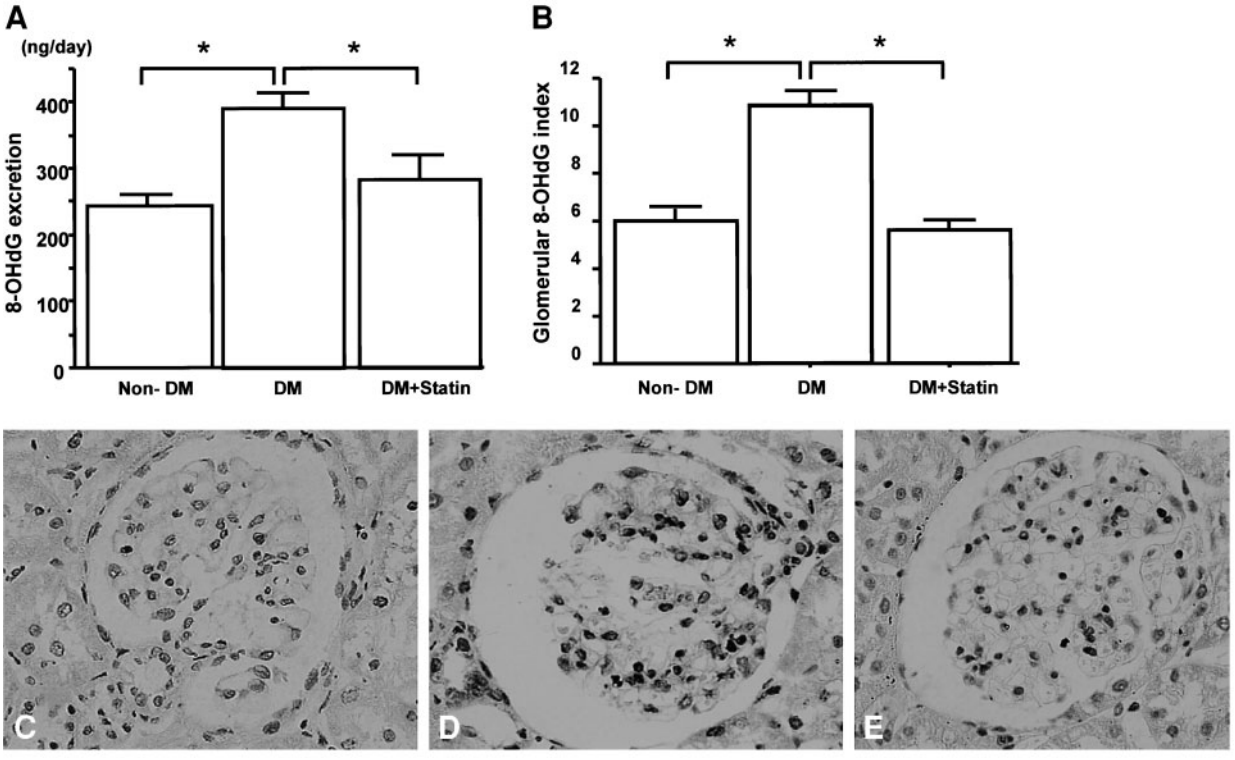

Fig. 5. Twenty-four-hour urinary 8-OHdG excretion (**A**) and glomerular 8-OHdG index (**B**) in non-diabetic controls (non-DM), untreated diabetics (DM) and statin-treated rats (DM + statin). Values are expressed as mean ± SEM. $^*P < 0.05$. Distribution of 8-OHdG in kidney tissues of non-diabetic controls (**C**), untreated diabetic (**D**) and statin-treated (**E**) groups. Accumulation of 8-OHdG was observed in glomerular and interstitial cells in the untreated diabetic group (D), but 8-OHdG accumulation was decreased in the statin-treated group (E).

statins inhibited NF-κB activation both *in vitro* and *in vivo* [6,13]. Our data suggest that cerivastatin may down-regulate ICAM-1 expression through inhibition of NF-κB activation.

Oxidative stress is a contributing factor in complications of diabetes mellitus. Oxidative stress can cause ICAM-1 up-regulation. Moreover, elevated oxidative stress is considered to increase the binding activities of the transcription factors NF-κB and AP-1. We evaluated oxidative stress measured by 8-OHdG, as it is one of the most established markers of oxidation [14]. We demonstrated that statin treatment reduced both glomerular deposition and urinary excretion of 8-OHdG in diabetic rats. Thus, statins are considered to have an anti-oxidative effect. Wagner *et al*. [15] demonstrated that statins inhibited super oxide anion formation in rat aorta by preventing the isoprenylation of p21 Rac. Our findings suggest that cerivastatin down-regulated NF-κB and ICAM-1 through reduction of oxidative stress in diabetic rats. We might reasonably interpret that statins could ameliorate the vicious circle of inflammation and oxidative stress.

In cultured endothelial cells or vascular smooth muscle cells, it has been demonstrated that statins can block the Rho GTPase-dependent mechanism, by blocking mevalonate pathway. Several studies indicate that cerivastatin prevents ICAM-1 expression or monocyte adhesion to the vascular endothelium via inhibition of Rho activity [16]. In mesangial cells, Danesh *et al*. [17] suggested statins might ameliorate the high glucose-induced mesangial proliferation by preventing Rho isoprenylation. Thus, inactivation of RhoA might be involved in the reduction of ICAM-1 expression and inactivation of NF-κB in our experiment.

Recently, statins have been shown to selectively block LFA-1-mediated adhesion and co-stimulation of lymphocytes. This effect is unrelated to the inhibition of HMG-CoA reductase; rather, it occurs via binding to a novel allosteric site within the LFA-1 molecule [18]. This unique effect might not be expected in our experiment because statins appear to exhibit this effect at much higher concentrations (μmol range) as compared with those administered *in vivo* (nmol range).

In addition to these anti-inflammatory effects, statin treatment led to decrease in blood pressure. Several reports approved that statins reduce blood pressure and improve endothelial function. These effects can be explained by down-regulation of angiotensin type 1 receptor, up-regulation of endothelial cell NO synthase (ecNOS) and consequent reduction of reactive oxygen species in vessel wall [19]. Anti-hypertensive effect might also contribute to the reduction of albuminuria and glomerular volume in our study.

There are many clinical studies to investigate the effects of ACEI on DN. However, there have been no clinical studies, which clarify the effects of statins on DN. Also, only a few studies have evaluated the effect of combination therapy with ACEI and statin in animal models. Zoja *et al*. [20] presented the data that single therapy with ACEI or statin had limited effects, but the combination therapy succeeded in further reduction of glomerulosclerosis and urinary protein excretion in rat model of Heymann nephritis. Combination therapy with ACEI and statins might be promising as an option for preventing renal injury.

In summary, statin treatment prevented glomerular injury in diabetic rats independent of cholesterol-lowering effects. Our data suggest that the beneficial effect might be mediated by pleiotropic effects including an anti-inflammatory action through a reduction of oxidative stress and the subsequent NF-κB activation, ICAM-1 expression and macrophage infiltration in the early phase of DN.

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