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Dipeptidyl peptidase-4 inhibitor ameliorates early renal injury through its anti-inflammatory action in a rat model of type 1 diabetes

Ryo Kodera [a,*], Kenichi Shikata [a,b], Tetsuharu Takatsuka [b], Kaori Oda [b], Satoshi Miyamoto [b], Nobuo Kajitani [b], Daisho Hirota [b], Tetsuichiro Ono [b], Hitomi Kataoka Usui [c], Hirofumi Makino [b]

[a] Center for Innovative Clinical Medicine, Okayama University Hospital, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan
[b] Department of Medicine and Clinical Science, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan
[c] Department of Primary Care and Medical Education, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan

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ABSTRACT

Introduction: Dipeptidyl peptidase-4 (DPP-4) inhibitors are incretin-based drugs in patients with type 2 diabetes. In our previous study, we showed that glucagon-like peptide-1 (GLP-1) receptor agonist has reno-protective effects through anti-inflammatory action. The mechanism of action of DPP-4 inhibitor is different from that of GLP-1 receptor agonists. It is not obvious whether DPP-4 inhibitor prevents the exacerbation of diabetic nephropathy through anti-inflammatory effects besides lowering blood glucose or not. The purpose of this study is to clarify the reno-protective effects of DPP-4 inhibitor through anti-inflammatory actions in the early diabetic nephropathy.

Materials and methods: Five-week-old male Sprague–Dawley (SD) rats were divided into three groups; non-diabetes, diabetes and diabetes treated with DPP-4 inhibitor (PKF275-055; 3 mg/kg/day). PKF275-055 was administered orally for 8 weeks.

Results: PKF275-055 increased the serum active GLP-1 concentration and the production of urinary cyclic AMP. PKF275-055 decreased urinary albumin excretion and ameliorated histological change of diabetic nephropathy. Macrophage infiltration was inhibited, and inflammatory molecules were down-regulated by PKF275-055 in the glomeruli. In addition, nuclear factor-κB (NF-κB) activity was suppressed in the kidney.

Conclusions: These results indicate that DPP-4 inhibitor, PKF275-055, have reno-protective effects through anti-inflammatory action in the early stage of diabetic nephropathy. The endogenous biological active GLP-1 might be beneficial on diabetic nephropathy besides lowering blood glucose.

1. Introduction

Diabetic nephropathy has become the most common cause of end-stage renal disease worldwide [1]. Many mechanisms have been proposed to explain the pathogenesis of diabetic nephropathy. Recently, accumulated data have emphasized that an inflammation plays a crucial role in the pathogenesis of diabetic nephropathy [2,3]. Actually, we have shown that inflammatory molecules and mediators are important in the early stage of diabetic nephropathy by using intercellular adhesion molecule-1 (ICAM-1) -deficient mice and macrophage scavenger receptor-A-deficient mice, and by an administration of methotrexate [4–6].

Currently, incretin-based drugs are being used to achieve better glycemic control in patients with type 2 diabetes. GLP-1 receptor agonists that enhance resistance to the degradation by DPP-4 enzyme, strongly and steadily stimulate GLP-1 receptor in the pharmacological level. On the other hand, DPP-4 inhibitors that block the activity of DPP-4, reinforce the endogenous biological actions of incretin.

The incretin receptors have been found in multiple organs including kidney [7]. Previous reports and our study [8] revealed that GLP-1 receptor was expressed in glomeruli and renal tubule. To our knowledge, gastric inhibitory polypeptide (GIP) receptor was not expressed in the kidney [9]. We studied the effects on diabetic nephropathy animal model by using GLP-1 receptor agonist called exendin-4. Exendin-4 reduced the urinary albumin

Abbreviations: DPP-4, dipeptidyl peptidase-4; GLP-1, glucagon-like peptide-1; SD, Sprague–Dawley; NF-κB, nuclear factor-κB; GIP, gastric inhibitory polypeptide; ICAM-1, intercellular adhesion molecule-1; PAM, periodic acid-methenamine silver; cAMP, cyclic AMP; CRP, c-reactive protein; IL, interleukin; CD, cluster of differentiation; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.

* Corresponding author. Fax: +81 86 235 6505.
E-mail address: kodera@cc.okayama-u.ac.jp (R. Kodera).

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excretion, and attenuated the histological parameters of glomerular injuries characterized by mesangial extracellular matrix expansion and glomerular hypertrophy. It also attenuated inflammatory molecules and mediators, such as ICAM-1, macrophage infiltration, cytokines and NF-κB activity, in the kidney. Furthermore, the effects were shown, observed directly through the GLP-1 receptor in culture cells [8]. The other groups also presented that GLP-1 receptor agonist was beneficial on diabetic nephropathy [10–12].

Recently, the other investigator reported that DPP-4 inhibitor ameliorated diabetic nephropathy by inhibition of apoptosis and sclerosis [13]. Therefore, the endogenous biological effects of GLP-1 would ameliorate renal injuries. However, it is not obvious whether DPP-4 inhibitor prevents the exacerbation of diabetic nephropathy through anti-inflammatory effects in the early stage model of diabetic nephropathy. The purpose of this study is to clarify the reno-protective effects of DPP-4 inhibitor through anti-inflammatory actions in the early diabetic nephropathy.

2. Methods

2.1. Animals

Male SD rats (Charles River, Yokohama, Japan) were purchased from Charles River (Yokohama, Japan). SD rats aged 4 weeks were divided into the following groups: negative control group, non-diabetes (NDM); positive control group, diabetes (DM); and test group, diabetes treated with DPP-4 inhibitor (DM + D) (n = 7 per group). At the age of 5 weeks, rats chosen for the DM and DM + D groups were injected intravenously with streptozotocin (Sigma–Aldrich, St. Louis, MO, USA) at 65 mg/kg body weight in citrate buffer (pH 4.5). Only rats with blood glucose concentrations >300 mg/dl at 7 days after streptozotocin injection were used in the diabetes groups. The NDM group received injections of citrate buffer alone. The DM + D group was given DPP-4 inhibitor (PKF275-055 [14]; Novartis, Basel, Switzerland) orally at 3 mg/kg body weight daily for 8 weeks, starting at 1 week after streptozotocin injection. All rats had free access to standard chow and tap water. All procedures were approved by the Committee for Ethics and Animal Experimentation of Nihon Bioresearch Inc. All rats were killed at 9th week after the induction of diabetes, and the kidneys were harvested.

2.2. Metabolic variables

Systolic blood pressure was measured by tail-cuff plethysmography (Softron, Tokyo, Japan). Food intake was calculated as the average over 3 days. Urine samples were collected over a 24 h period in individual metabolism cages. Urinary albumin excretion was measured by nephelometry using anti-rat albumin antibody (ICN Pharmaceuticals, Aurora, OH, USA). Creatinine clearance (ml min^{-1} kg^{-1}) was calculated as described previously [15]. Serum active GLP-1 levels were measured by using ELISA kit (AKMGP-011, Shibayagi, Gunma, Japan) at the pre-prandial (after about 12 h of fasting) and post-prandial (2 h) time. 24 h urinary cyclic AMP (cAMP) excretion was measured by using cAMP Complete Enzyme Immunometric Assay kit (Enzo Life Sciences, Ann Arbor, USA) according to the manufacturer's instructions. Serum c-reactive protein (CRP) levels were measured by rat CRP ELISA kit (Life Diagnostics, PA, USA) according to the manufacturer's instructions. Serum interleukin (IL)-6 levels were measured by rat IL-6 ELISA kit (Uscn Life Science, China) according to the manufacturer's instructions.

2.3. Light microscopy

Periodic acid-methenamine silver (PAM)-stained slice were analyzed as described previously [8]. To evaluate the glomerular size and mesangial matrix area, we examined randomly selected ten glomeruli per animal (n = 7 per group). Quantitative analysis for all staining was performed in a blinded manner.

2.4. Immunoperoxidase staining

Immunoperoxidase staining was performed as described previously [16]. Primary antibody was monoclonal antibody against rat monocytes/macrophages (ED1, 1:50; Serotec, Oxford, UK), which was applied for 12 h at 4 °C. Secondary antibody was biotin-labelled goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA) for 60 min at room temperature. Intraglomerular ED1-positive cells were counted in ten glomeruli per animal (n = 7 per group). Quantitative analysis for all staining was performed in a blinded manner.

2.5. RNA extraction, quantitative real-time PCR

Total RNA was extracted from glomeruli isolated by the mechanical sieving technique as previously reported [17], and by using a kit (RNeasy plus Mini; Qiagen, Valencia, CA, USA). Real-time PCR was performed as described previously [8]. The amount of PCR products was normalized with β-actin. The specific oligonucleotide primer sequences are shown in Supplementary Table S1.

2.6. Cytokines and chemokines in the kidney

High-throughput multiplex immunoassays in the renal cortex were performed with the Procarta cytokine assay kit (Panomics Inc., CA, USA) according to the manufacturer's instructions, and analyzed by using Bio-plex (Bio-Rad, Tokyo, Japan).

2.7. Nuclear factor-κB activity

Nuclear proteins were extracted from the kidney tissues with a nuclear extract kit (Active motif, Carlsbad, CA) according to the manufacturer's instructions. NF-κB p65-dependent DNA-binding activity was determined by TransAM NF-κB p65 (Active motif) according to the manufacturer's instructions.

2.8. Statistical analysis

All values are expressed as the means ± SEM. Differences between groups were examined for statistical significance by using one-way ANOVA followed by Scheffe's test. For comparisons between two groups, an un-paired t test was used to assess statistical significance. A P value < 0.05 was considered statistically significant.

3. Results

3.1. Metabolic characteristics of experimental animal models

At 8th week after induction of diabetes, HbA1c, food intake, creatinine clearance and kidney weight were elevated to the same level in both the diabetic groups compared with the NDM group (Table 1). Body weight was decreased to the same level in both the diabetic groups. However, there was no significant difference between the DM and DM + D groups. Systolic blood pressure remained at the same level in the three groups. It is noteworthy that PKF275-055 treatment significantly reduced urinary albumin excretion compared with the DM group.

In this experimental animal model, serum active GLP-1concentration was significantly increased in the DM + D group compared with the NDM group at both the pre-prandial and post-prandial

Table 1
Physiological and metabolic characteristics of non-diabetes, diabetes and diabetes treated with DPP-4 inhibitor, PKF275-055, at the 8th week.

	NDM	DM	DM+D
HbA1c (%)	4.4 ± 0.1**	9.9 ± 0.5	9.7 ± 0.2
Body weight (g)	449 ± 13.3**	254 ± 16.2	260 ± 16.7
Food intake (g/day)	24.9 ± 0.9**	49.0 ± 1.5	51.7 ± 3.5
Systolic blood pressure (mmHg)	135 ± 2	133 ± 4	130 ± 2
Urinary albumin excretion (μg/day)	135 ± 24.4##	699 ± 48.7	420 ± 93.3#
Creatinine clearance (ml/min/kgBW)	7.6 ± 0.2*	12.6 ± 1.5	12.5 ± 1.3
Kidney weight (g/kgBW)	3.7 ± 0.1**	7.2 ± 0.6	7.7 ± 0.6

NDM, non-diabetic group; DM, diabetes control; DM + D, diabetes treated with PKF275-055; BW, body weight. Values are presented as means ± SEM. $n = 7$ per group.
* $P < 0.05$.
** $P < 0.001$ vs DM, DM + D.
# $P < 0.05$.
$P < 0.001$ vs DM.

time. There was significant difference at only the post-prandial time between the DM and DM + D groups (Fig. 1A).

To examine the association with the GLP-1 receptor signal pathway, we measured urinary cAMP excretion known as the second messenger of GLP-1 receptor. Urinary cAMP excretion was significantly elevated in the DM + D group compared with the other groups (Fig. 1B).

3.2. DPP-4 inhibitor ameliorated glomerular morphology in the early stage of diabetic nephropathy

Glomerular hypertrophy was observed in both the diabetic groups as compared with the NDM group. There was no significant difference in glomerular size between the DM and DM + D groups (Fig. 2D). Mesangial matrix expansion was also observed in both the diabetic groups as compared with the NDM group. However, PKF275-055 treatment significantly prevented mesangial matrix expansion compared with the DM group. The prevention of mesangial matrix expansion was not at the same level to the NDM group (Fig. 2E).

3.3. The reno-protective effects of DPP-4 inhibitor through anti-inflammatory action

To evaluate the anti-inflammatory effect of DPP-4 inhibitor in the glomeruli, we examined inflammatory-related factor. The

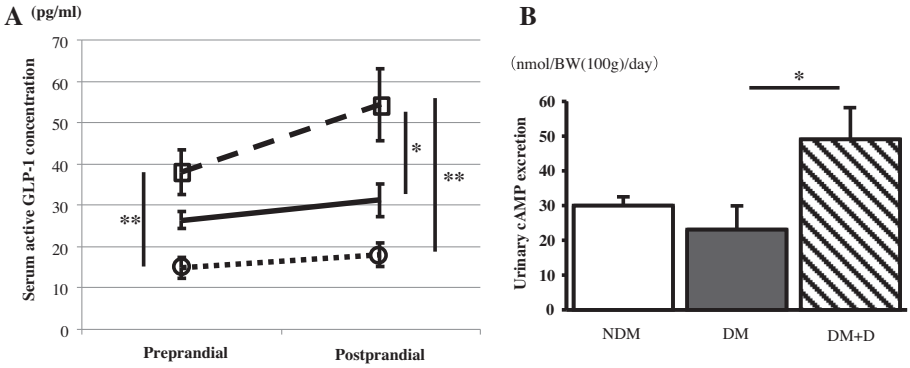

Fig. 1. Serum active GLP-1 concentration and urinary cAMP excretion in this model. (A) The concentration of the serum active GLP-1 at the preprandial and postprandial condition. Data are means ± SEM $n = 7$ per group. Dotted line (○), NDM; solid line, DM; Dotted line (□), DM + D. *$P < 0.05$; **$P < 0.001$. (B) Urinary cAMP excretion for 24 h. Data are means ± SEM $n = 7$ per group. *$P < 0.05$. NDM, non-diabetic group; DM, diabetes control; DM + D, diabetes treated with PKF275-055.

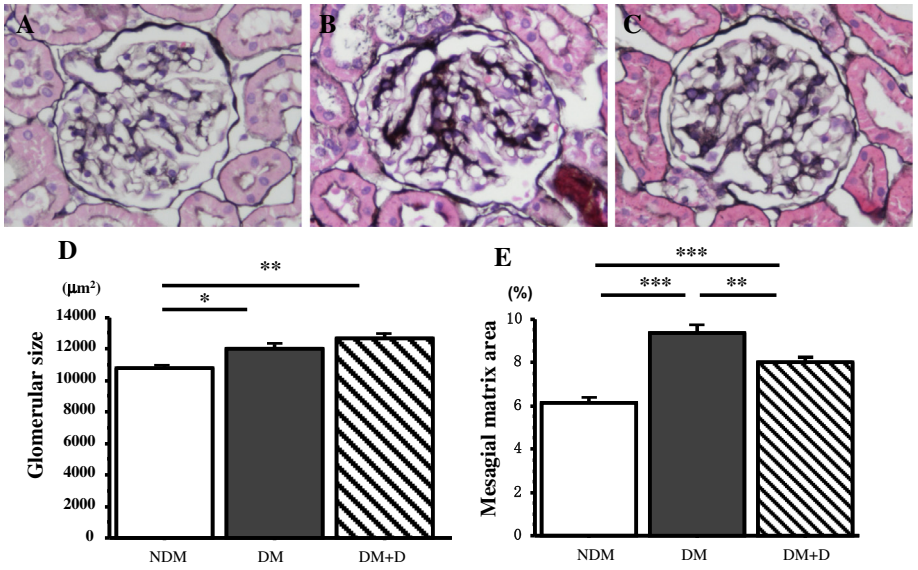

Fig. 2. The effects of DPP-4 inhibitor against glomerular morphology. Periodic acid-methenamine silver (PAM)-stained slice in glomeruli. (A) non-diabetic group, (B) diabetes control, (C) diabetes treated with PKF275-055. Randomly selected ten glomeruli per animal ($n = 7$ per group) were measured the glomerular size (D) and mesangial matrix area (E). Quantitative analysis for all staining was performed in a blinded manner. Data are means ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. NDM, non-diabetic group; DM, diabetes control; DM + D, diabetes treated with PKF275-055.

average number of macrophages (ED-1 positive cells) per glomerulus was markedly increased in the DM group compared with the NDM group, whereas macrophage infiltration was significantly inhibited by PKF275-055 treatment (Fig. 3A–D). Likewise, the mRNA level of cluster of differentiation (CD) 68 (a cell surface marker of macrophages) was significantly up-regulated in the DM group compared with the NDM group, and then significantly down-regulated in the DM + D group (Fig. 3E).

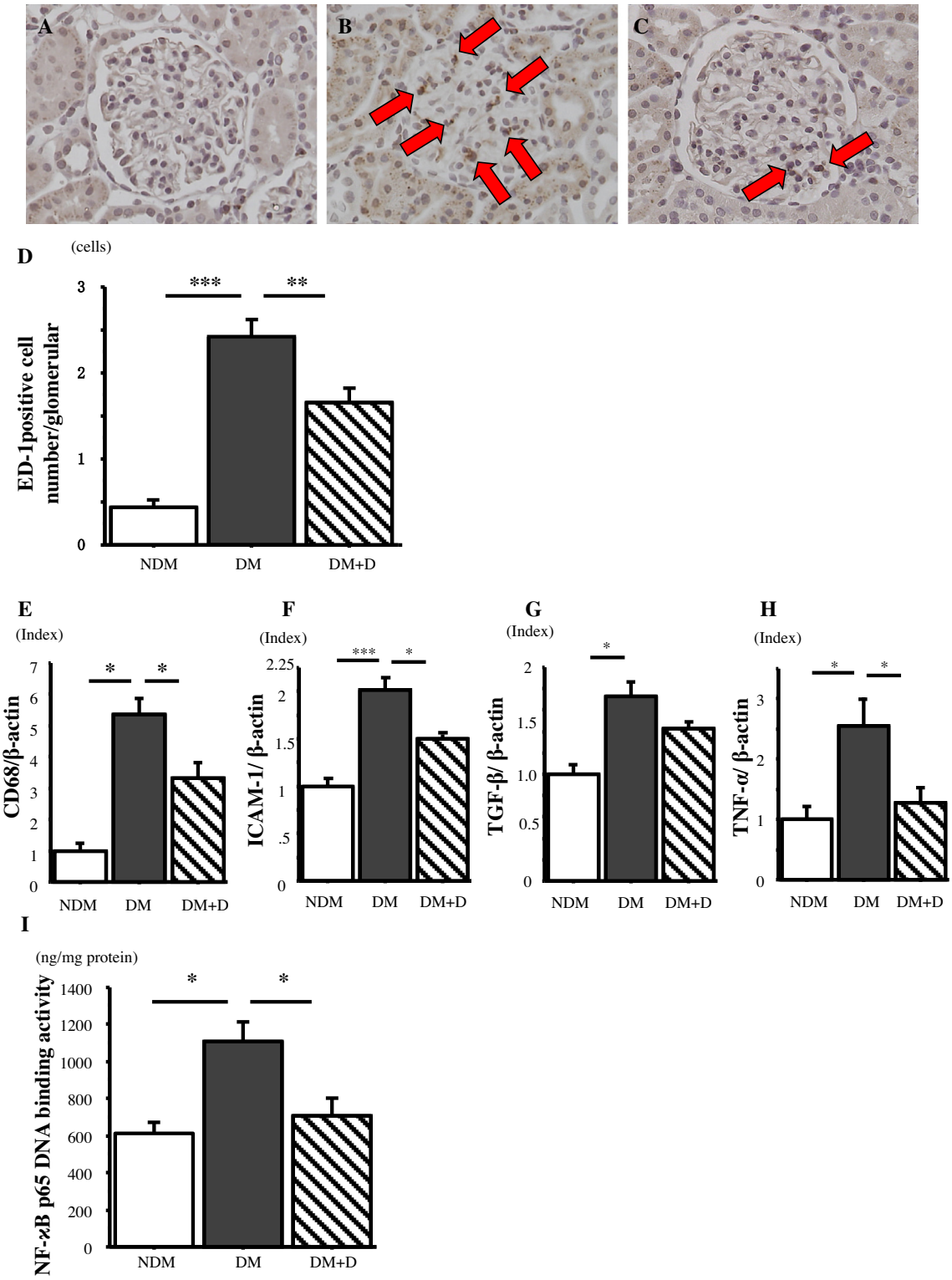

Fig. 3. The anti-inflammatory effects of DPP-4 inhibitor. (A–C) Immunoperoxidase staining for macrophage infiltration into glomeruli. (A) non-diabetic group, (B) diabetes control, (C) diabetes treated with PKF275-055. Arrow head is presented as macrophage. (D) Randomly selected ten glomeruli per animal (n = 7 per group) were measured the number of macrophages per glomerulus. Quantitative analysis for all staining was performed in a blinded manner. (E–H) The mRNA levels in the glomeruli (n = 6 per group). Values (means ± SEM) are presented as the ratio of NDM. (I) The NF-κB p65 DNA binding activity in the kidney. n = 5 per group. Absorbance was normalized to milligram protein. Data are means ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. NDM, non-diabetic group; DM, diabetes control; DM + D, diabetes treated with PKF275-055.

In addition, the mRNA levels of ICAM-1, transforming growth factor (TGF)-β and tumor necrosis factor (TNF)-α were significantly up-regulated in the DM group as compared with the NDM group. PKF275-055 treatment significantly down-regulated the mRNA levels of ICAM-1 and TNF-α compared with the DM group (Fig. 3F–H).

To evaluate the effect of DPP-4 inhibitor against the NF-κB activity, we examined NF-κB p65-dependent DNA-binding activity in the kidney. The NF-κB p65-dependent DNA-binding activity was significantly increased in the DM group compared with the NDM group. PKF275-055 treatment significantly decreased the NF-κB p65-dependent DNA-binding activity (Fig. 3I).

In order to clarify the influence of cytokines and chemokines which are cleaved by DPP-4 enzyme, we examined the production of eotaxin, Il-2, IL-1β, IP-10, MCP-1, MCP-3 and RANTES in the cortex. These cytokines and chemokines were not affected by PKF275-055 treatment (Supplementary Fig. S1 A–G).

To evaluate systemic inflammation, we measured the levels of serum CRP and IL-6. The level of serum CRP was significantly elevated in the DM group compared with the NDM group, but not attenuated by PKF275-055 treatment. The level of serum IL-6 was not affected (Supplementary Table S2).

4. Discussion

Our results indicated that DPP-4 inhibitor, PKF275-055, reduced urinary albumin excretion, and histological change of diabetic nephropathy, and prevented the macrophage infiltration and inflammatory molecules in the glomeruli. In addition, it inhibited the NF-κB activity in the kidney. DPP-4 inhibitor treatment didn't have an impact on glucose control, blood pressure and body weight which affect nephropathy. These results demonstrated that DPP-4 inhibitor ameliorated diabetic nephropathy through anti-inflammatory effects.

We should consider the following contents against the anti-inflammatory action of DPP-4 inhibitor. (1) The increase of active GLP-1 and GIP, (2) the inhibition of DPP-4 itself, (3) the influence of the other substrate of DPP-4 except for an incretin.

In terms of the influence of active GLP-1 and GIP, several previous reports have already shown that GLP-1 has reno-protective effects through anti-inflammation and anti-oxidative stress [8,10,11]. On the other hand, GIP receptor is not expressed in the kidney to our knowledge [9]. Therefore, it would be unlikely about the effects through GIP.

Recently, the inhibition of DPP-4 itself suppressed the secretion of cytokine from macrophage and NF-κB activity induced by lipopolysaccharide [18]. Therefore, the inhibition of DPP-4 itself might ameliorate diabetic nephropathy through anti-inflammatory effect by DPP-4 inhibitor. It is not directly observed in this study. Thereafter, it is necessary to examine it by using tissue-specific DPP-4 deficient animal model.

We examined the influence of cytokines and chemokines which are cleaved by DPP-4 enzyme. There are eotaxin, IL-2, IL-1β, IP-10, MCP-1, MCP-3 and RANTES as the substrate of DPP-4 related to inflammation. As for our result, at least DPP-4 inhibitor did not significantly aggravate inflammation in the kidney.

A better glycemic control prevents the onset and progression of diabetic nephropathy. In the previous report, DPP-4 inhibitor actually ameliorated glucose control in patients with type 2 diabetes [19] and animal model [20], and resulted in reducing urinary albumin excretion. Decreases of blood pressure and body weight are also useful for nephropathy. Incretin-based drugs have the inhibitory action of sodium reabsorption in renal proximal tubule under the conditions of salt-sensitive models and salt load [21,22]. However, it is considered that such action was not observed in the condition of this study. There was also no influence in body weight. It does not contradict with the clinical data [23].

Liu and the members [13] administrated DPP-4 inhibitor, LAF237 (vildagliptin), to same nephropathy model and suppressed urinary albumin, and reduced renal injury. In their examination, several parameters were evaluated at 12th and 24th week after the treatment. The stage was the period of drop of creatinine clearance. On the other hand, our examination at 8th week was at the more early stage, which indicated glomerular hyperfiltration. To our knowledge, our examination is the first report that DPP-4 inhibitor has protective effect at the early stage. Their results indicated that DPP-4 inhibitor suppressed urinary 8-Hydroxydeoxyguanosine excretion known as the marker of oxidative stress. Our result was not the same, which DPP-4 inhibitor could not significantly reduce the oxidative stress elevated by the hyperglycemia (data not shown). The longer period of DPP-4 inhibitor treatment might be required to decrease the oxidative stress.

In our previous report on GLP-1 receptor agonist [8], there were improvements on glomerular hyperfiltration in the same stage of nephropathy model, but the same result did not appear in this study. This reason is considered to be due to the difference between the pharmacological effect expected by GLP-1 receptor agonist and the biological effect expected by DPP-4 inhibitor. According to the previous report examining about the inhibitory action of sodium reabsorption in the kidney between GLP-1 receptor agonist and DPP-4 inhibitor, GLP-1 receptor agonist elevated more urinary cAMP concentration than DPP-4 inhibitor, and the effect of it depended on the cAMP levels[24]. However, the details are still unclear whether GLP-1 signal contributes to glomerular hemodynamics or not. Therefore, a further study is necessary about it.

The serum CRP level was not improved by DPP-4 inhibitor in this study. The result caused a question about whether PKF275-055 treatment in this study affects the systemic inflammation. We proposed the difference of the DPP-4 expression in each organ. The previous report [25] described that DPP-4 activity was strongly expressed in the kidney compared with the other tissues. Furthermore, the prevention of DPP-4 itself by DPP-4 inhibitor attenuated the local inflammation [18]. Therefore, the anti-inflammatory effect on kidney through DPP-4 inhibitor might be more affected compared with whole body. In addition, the long-term treatment might be necessary to decrease the systemic inflammation similarly to oxidative stress. In our previous study on GLP-1 receptor agonist [8], oxidative stress was attenuated through the same treatment period, and in the same animal model. Taken together, the anti-inflammatory effect expected by PKF275-055 treatment might be weaker than GLP-1 receptor agonist. However, further examination is necessary to investigate this regard.

In conclusion, we showed that DPP-4 inhibitor, PKF275-055, have reno-protective effects through anti-inflammatory action in the early stage of diabetic nephropathy. The endogenous biological active GLP-1 might be beneficial on diabetic nephropathy besides lowering blood glucose.

Conflict of interest statement

This work was partially supported by research fund from Novartis Pharma K.K. KS receives speaker honoraria from Astellas, MSD, Eli Lilly Japan, Novartis Pharma, NovoNordisk, Ono, Sanofi and Tanabe Mitsubishi, and receives grant support from Tanabe Mitsubishi. HM is a consultant for AbbVie, Astellas, and Teijin, receives speaker honoraria from Astellas, MSD, Takeda, and Tanabe Mitsubishi, and receives grant support from Astellas, Daiichi Sankyo, Dainippon Sumitomo, MSD, Novo Nodisk, Takeda and Kyowahakko-Kirin.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.049

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