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Original Article

Tissue level of advanced glycation end products is an independent determinant of high-sensitivity C-reactive protein levels in haemodialysis patients

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KEY WORDS:
advanced glycation end products, atherosclerosis, haemodialysis, inflammation.

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SUMMARY AT A GLANCE

This study demonstrates the association between tissue advanced glycation end products (AGE) level and high-sensitivity C-reactive protein (hsCRP) in haemodialysis patients. In addition, tissue AGE and hsCRP levels could in concert contribute to the progression of atherosclerosis in these subjects.

ABSTRACT:

Aim: **C-reactive protein (CRP) level predicts future cardiovascular events in patients on haemodialysis (HD). Advanced glycation end products (AGE) play a role in cardiovascular disease (CVD) in HD patients. However, which variables including tissue AGE levels are independently associated with CRP remains unknown. Therefore, whether tissue AGE and CRP levels were correlated with atherosclerosis in HD patients was examined.**
Methods: **Fifty-four HD patients underwent determinations of blood chemistries and tissue AGE. Tissue AGE levels were evaluated by measuring skin autofluorescence. Pulsatility index (PI) in the carotid artery was measured using a Doppler ultrasonography.**
Results: **Univariate analyses showed that age, white blood cells, serum albumin (inversely), alkaline phosphatase (inversely), tartrate-resistant acid phosphatase 5b (TRAP5b) (inversely) and skin AGE levels were significantly correlated with high-sensitivity CRP (hsCRP). Multiple stepwise regression analysis revealed that serum albumin, TRAP5b and skin AGE levels were independent determinants of hsCRP. Further, PI was highest among HD patients with high skin AGE and high hsCRP levels.**
Conclusion: **The present study suggests that tissue AGE level is one of the independent determinants of hsCRP in HD patients. Tissue AGE and hsCRP levels may be correlated with each other, which could in concert contribute to the progression of atherosclerosis in these subjects.**

Cardiovascular disease (CVD) is a major cause of morbidity and high mortality rates in patients on haemodialysis (HD).[1,2] Recently, chronic inflammation is shown to be involved in accelerated atherosclerosis, which could lead to increased risks of CVD in these high-risk patients.[3] Indeed, several epidemiological studies have shown that an inflammatory biomarker, C-reactive protein (CRP) level, is elevated in patients with HD, which predicts future cardiovascular events and death.[4] Although various metabolic and haemodynamic derangements are supposed to cause chronic inflammation in HD patients,[5–7] which factors are independently associated with CRP level is not fully understood, because these variables are correlated with each other and associated with inflammation.[8,9]

Reducing sugars can react non-enzymatically with the amino groups of proteins to initiate a complex series of rearrangements and dehydrations, and then to produce a class of irreversibly cross-linked, fluorescent moieties termed advanced glycation end products (AGE).[10–12] Recently, the formation and/or accumulation of AGE have been known to progress in a normal aging process, and at an accelerated rate under diabetes or chronic kidney disease, thus playing a role in the development and progression of a CVD in these subjects.[13–18] Because AGE are reported to elicit oxidative stress generation and vascular inflammation in both cell culture and animal models, it is conceivable that accumulation of AGE is one of the causative factors for the elevation of CRP levels in patients with HD.

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Many AGE form molecular cross-links and fluorescence *in vivo*.[19,20] Therefore, tissue AGE levels could be evaluated non-invasively by measuring skin autofluorescence (AF). Indeed, skin AF value has been shown to be associated with vascular complications in diabetes and predict cardiovascular mortality in patients with HD.[19,20] In this study, we investigated which anthropometric and metabolic variables, including tissue AGE levels which were evaluated quantitatively by measuring skin AF with an AGE reader, were independently associated with CRP in HD patients. We further examined whether tissue AGE and CRP levels were correlated with atherosclerosis in these subjects.

METHODS

Patients

Fifty-four consecutive outpatients on HD (32 men and 22 women; mean age 54.2 ± 14.0 years) underwent a complete history and physical examination, determinations of blood chemistries, anthropometric and metabolic variables. Mean duration of HD was 7.9 ± 5.1 years. Patients were dialyzed for 5 h with high-flux dialyzers three times a week. Nine patients had diabetes mellitus (DM). Thirteen patients had angiographically proven CVD and/or a history of coronary heart disease. The remainders had no history of CVD. Forty-one patients received inhibitors of the renin–angiotensin system, and only two patients received statins for the treatment of dyslipidaemia. We excluded any patients with inflammatory, neoplastic disorders and those who had a recent (<3 months) acute coronary syndrome, stroke and any acute infection.

Data collection

The medical history was ascertained by a questionnaire. Height and weight were measured, and body mass index (BMI) (kg/m^2) was calculated as an index of presence or absence of obesity. Blood pressure (BP) was measured in the sitting position using an upright standard sphygmomanometer. Vigorous physical activity and smoking were avoided for at least 30 min before BP measurement.

Blood was drawn from arteriovenous shunt just before starting HD for determinations of lipids (total cholesterol, low-density lipoprotein (LDL) cholesterol and triglycerides), haemoglobin, haemoglobin A1c (HbA1c), albumin, blood urea nitrogen (BUN), creatinine (Cr), uric acid, Ca, P and Fe. Intact parathyroid hormone (PTH) was evaluated by an immunoradiometric assay (IRMA, Allegro Intact PTH; Nichols Institute, San Juan Capistrano, CA, USA). Tartrate-resistant acid phosphatase 5b (TRAP5b) activity, a bone resorption marker unaffected by renal function, was measured by a novel fragment-absorbed immunocapture enzymatic assay (FAICEA) as described previously.[21] High-sensitivity CRP (hsCRP) was measured with nephelometry (N-Latex, CRPII; Dade Behring, Tokyo, Japan). The other chemistries were measured at a commercially available laboratory as described previously (Wako Pure Chemical Industries, Osaka, Japan). HD adequacy was evaluated by a single-pool fractional clearance of body water for urea (Kt/V).[22] Single-pool Kt/V was calculated by the following formula:

$$Kt/V = -\ln(Ce/Cs - 0.008 \times t) + (4 - 3.5 \times Ce/Cs) \times \Delta BW/BW \quad (1)$$

where Ce/Ce is post-serum/pre-serum urea nitrogen ratio, t is dialysis time, ΔBW/BW is the ratio of the ultrafiltrate volume removed from the post-dialysis weight and ln is a natural logarithmic (ln) transformation.

Informed consent was obtained from all patients, and the study protocol was approved by the Institutional Ethics Committee of Kurume University School of Medicine.

Pulsatility index (PI) in the carotid artery, a surrogate marker of atherosclerosis, was evaluated by high-resolution ultrasonography with a 7.5 MHz linear probe (GE Yokogawa Medical Systems, Tokyo, Japan). Common carotid PI was measured as a reflection of impedance to flow distal to the point of sampling.[23] PI was automatically calculated and indicated as ((peak systolic velocity) – (end diastolic velocity)) / mean flow velocity.

Tissue AGE levels were evaluated quantitatively by measuring skin AF with an AGE reader according to the supplier's recommendations (DiagOptics BV, Groningen, the Netherlands).[20]

Statistical analysis

Results are presented as mean ± standard deviation (SD). The medications for hypertension and dyslipidaemia (renin–angiotensin system (RAS) inhibitors and statins) and the presence or absence of DM were coded as dummy variables. Because of their skewed distributions, a natural logarithmic (ln) transformation was performed for hsCRP. Univariate analysis was performed for determinants of hsCRP. To determine independent determinants of hsCRP and tissue AGE, multiple stepwise regression analysis was performed. PI levels stratified by tissue AGE and hsCRP levels were compared using unpaired Student's *t*-test. Statistical significance was defined as $P < 0.05$. All statistical analyses were performed with SPSS software (SPSS, Chicago, IL, USA).

RESULTS

Demographic data are shown in Table 1. Univariate analyses showed that age ($\beta = 0.298$, $P = 0.03$), white blood cells (WBC) ($\beta = 0.294$, $P = 0.03$), serum albumin ($\beta = -0.506$, $P < 0.001$), alkaline phosphatase (ALP) ($\beta = -0.275$, $P = 0.04$), TRAP5b ($\beta = -0.412$, $P = 0.002$) and skin AGE levels ($\beta = 0.378$, $P = 0.005$) were significantly correlated with serum levels of hsCRP (Table 2). Because these significant parameters could be closely correlated with each other, multiple regression analysis was performed. Multiple stepwise regression analysis revealed that serum albumin ($\beta = -0.431$, $P < 0.001$), TRAP5b ($\beta = -0.390$, $P < 0.001$) and skin AGE levels ($\beta = 0.308$, $P = 0.004$) were independent determinants of hsCRP (Table 2). We also found that the presence of DM ($\beta = 0.385$, $P = 0.002$) and hsCRP ($\beta = 0.379$, $P = 0.002$) were independently associated with skin AGE levels in our subjects.

Further, we found that hsCRP was significantly correlated with PI ($\beta = 0.396$, $P = 0.003$) and that hsCRP was one of the independent determinants of PI in our patients ($R^2 = 0.311$, $P = 0.009$).

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Table 1 Clinical characteristics of the patients

No. of patients	54
Age (years)	54.2 ± 14.0
Sex (*n*) (male/female)	32/22
Body mass index (kg/m²)	20.8 ± 3.53
Systolic blood pressure (mmHg)	134.0 ± 21.1
Diastolic blood pressure (mmHg)	80.0 ± 12.2
Haemoglobin (g/dL)	11.4 ± 1.25
Albumin (g/dL)	3.60 ± 0.35
BUN (mg/dL)	59.9 ± 11.1
Serum Cr (mg/dL)	11.2 ± 2.36
Uric acid (mg/dL)	7.40 ± 1.08
Ca (mg/dL)	9.53 ± 0.86
P (mg/dL)	5.45 ± 1.11
Fe (mg/dL)	66.8 ± 22.7
Total cholesterol (mg/dL)	159.5 ± 34.9
LDL cholesterol (mg/dL)	82.1 ± 28.1
Triglycerides (mg/dL)	127.6 ± 94.9
ALP (U/L)	17.9 ± 9.32
Intact PTH (pg/mL)	71.8 ± 52.0
TRAP5b (U/L)	384.2 ± 175.3
Log hsCRP	2.89 ± 0.72
HbA1c (%)	5.09 ± 0.87
Diabetes (*n*) (–/+)	45/9
PI	2.04 ± 0.73
HD duration (years)	7.9 ± 5.1
Kt/V	1.77 ± 0.33
Skin AGE (a.u.)	2.84 ± 0.71
Medication	
RAS inhibitors (*n*) (–/+)	13/41
Statins (*n*) (–/+)	52/2

Values are shown as mean ± standard deviation. AGE, advanced glycation end products; ALP, alkaline phosphatase; a.u., arbitrary units; BUN, blood urea nitrogen; HbA1c, haemoglobin A1c; HD, haemodialysis; hsCRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; PI, pulsatility index; PTH, parathyroid hormone; RAS, renin–angiotensin system; TRAP5b, tartrate-resistant acid phosphatase 5b.

Then, in order to examine the relationships among skin AGE, hsCRP and carotid PI, we divided the study participants into four groups according to their AGE (low AGE, ≤2.84; high AGE, >2.85) and hsCRP (low hsCRP, log ≤2.42; high hsCRP, log >2.43) levels. As shown in Figure 1, carotid PI was found to be highest among HD patients with high skin AGE and high hsCRP levels.

DISCUSSION

The salient findings of this study are: (i) tissue AGE levels evaluated by skin AF with a specific AGE reader is one of the independent determinants of hsCRP levels in HD patients; (ii) besides the presence of DM, hsCRP is a sole determinant of skin AGE levels; and (iii) carotid PI, a surrogate marker of atherosclerosis, is highest among HD patients with high tissue AGE and high hsCRP levels. Therefore, our present study suggests that: (i) tissue accumulation of AGE might be one of the causative factors for the elevation of hsCRP in HD patients; and (ii) tissue AGE and CRP are correlated with each other, both of which could in concert contribute to the progression of atherosclerosis in patients with HD.

In this study, we evaluated tissue AGE levels by measuring skin AF because: (i) measurement of skin AF with a commercially available AGE reader is a non-invasive, reliable and time-saving method to estimate tissue accumulation of AGE;[3] (ii) skin AF values are reported to significantly correlate with skin AGE levels evaluated by high-performance liquid chromatography (HPLC) in both diabetic and non-diabetic subjects;[20] and (iii) skin AF was positively associated with the severity of vascular complications in patients with both type 1 and type 2 diabetes mellitus and a predictor of future cardiovascular events and death in patients with HD.[19,20] Skin AF levels in our HD patients were 2.84 ± 0.71, whose value was 1.6-fold higher than that in age- and sex-matched Japanese controls.[24] The results were consistent with the previous findings of Arsov *et al.*, who showed that skin AF values were increased by approximately 1.4-fold in HD patients compared with controls.[25] These observations further support the concept that measurement of skin AF with an AGE reader is a reliable diagnostic tool to evaluate tissue AGE accumulation in HD patients.

This study was cross-sectional one and thus could not assess the questions of whether elevation of tissue AGE level was a cause or consequence of chronic inflammation. However, we have previously shown that AGE could induce CRP production by hepatoma cells through oxidative stress and inflammatory reactions.[26] Further, inflammation is shown to cause oxidative stress generation, which could lead to promotion of the formation and accumulation of AGE.[16] These observations suggest that decreased renal clearance of AGE in HD patients could contribute to enhanced tissue accumulation of AGE and subsequently evoke inflammatory reactions, which may in turn further stimulate the formation and accumulation of AGE. In other words, AGE accumulation and inflammatory reactions may be correlated with each other in patients with HD, which could account for the positive correlations between skin AF and hsCRP levels in our subjects. Tan *et al.*[27] reported that serum AGE levels were independently associated with CRP in patients with type 2 diabetes as well.

In the present study, PI, a surrogate marker of atherosclerosis, which reflects the degree of vascular resistance,[25,28] is highest among HD patients with high skin AGE and high hsCRP levels. Nakatou *et al.* have demonstrated that the PI is significantly correlated with the atherosclerosis risk score and the existence of cerebral infarction in diabetic patients.[28] There is a growing body of evidence that AGE could elicit vascular inflammation and thrombogenesis, playing a central role in atherosclerosis. Further, recently, CRP has been shown to evoke endothelial cell damage, pro-inflammatory reactions and smooth muscle cell proliferation, thereby being involved in the pathogenesis of CVD as well. An enhanced positive feedback loop between tissue AGE accumulation and CRP induction as described above could in concert contribute to the development and progression of atheroscle-

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Table 2 Univariate and multiple stepwise regression analysis for the correlates of log hsCRP

Variables	Univariate			Multiple stepwise regression		
	β	SE	*P*-value	β	SE	*P*-value
Age	**0.298**	**0.007**	**0.029**			
Sex	0.033	0.2	0.811			
Systolic BP	0.1	0.005	0.471			
WBC	**0.294**	**0**	**0.031**			
Haemoglobin	–0.183	0.078	0.185			
ALP	**–0.275**	**0.01**	**0.044**			
Albumin	**–0.506**	**0.244**	**<0.001**	**–0.431**	**0.208**	**<0.001**
BUN	0.041	0.009	0.771			
Uric acid	0.054	0.092	0.697			
Total cholesterol	0.105	0.003	0.456			
LDL cholesterol	0.124	0.004	0.377			
Intact PTH	–0.139	0.001	0.315			
TRAP5b	**–0.412**	**0.001**	**0.002**	**–0.39**	**0**	**<0.001**
Skin AGE	**0.378**	**0.13**	**0.005**	**0.308**	**0.103**	**0.004**
Kt/V	–0.146	0.296	0.293			
DM	–0.002	0.264	0.991			
CVD	0.188	0.226	0.174			

Bolded text indicates statistically significant values. β, standardized regression coefficients. $r^2 = 0.495$. ALP, alkaline phosphatase; BP, blood pressure; BUN, blood urea nitrogen; CVD, cardiovascular disease; DM, diabetes mellitus; hsCRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; PTH, parathyroid hormone; TRAP5b, tartrate-resistant acid phosphatase 5b; SE, standard error; WBC, white blood cell.

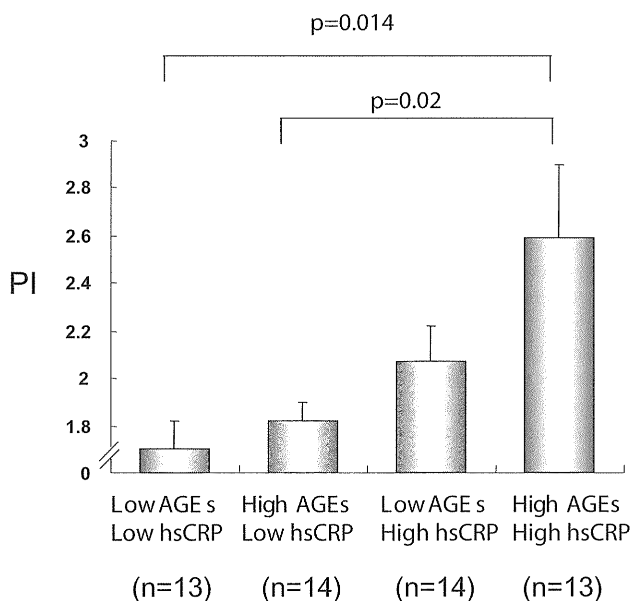

Fig. 1 Relationships among skin AGE, hsCRP and carotid PI in HD patients. AGE, advanced glycation end products; hsCRP, high-sensitivity C-reactive protein; PI, pulsatility index.

rosis in HD patients. However, it should be noted that Mulder *et al.* reported that skin AF values were elevated in patients with stable coronary artery disease, but not correlated with CRP levels.[29] We did not know the exact reasons for the discrepant results between ours and theirs. The difference of subject population and ethnicity could account for the discrepancy.

In this study, low serum albumin and TRAP5b levels were also independently correlated with hsCRP. There is accumulating evidence to show that malnutrition is involved in inflammation and atherosclerosis in chronic kidney disease patients.[30] Further, chronic inflammation is shown to impair bone turnover in HD patients.[31] The findings suggest that low serum albumin and low TRAP5b levels could reflect malnutrition and impaired bone turnover in our patients, respectively, which could explain the link between high hsCRP and low serum albumin or TRAP5b levels.

The study was a cross-sectional non-intervention one. Therefore, it did not elucidate the causative relationships between tissue AGE and hsCRP levels. However, as described above, AGE are reported to stimulate CRP production by the liver *in vitro*.[28] So, it is conceivable that tissue AGE accumulation could be one of the causative factors for the elevation of hsCRP in HD patients. Tissue AGE accumulation and hsCRP elevation may act in concert to promote atherosclerosis in these patients. Longitudinal and/or interventional studies are needed to clarify whether suppression of AGE formation could decrease tissue AGE and hsCRP levels and subsequently reduce the risk of future cardiovascular events in HD patients.

In this study, we measured tissue AGE levels by evaluating skin AF with an AGE reader. However, we have to say that there are some limitations of this method: (i) not only tissue AGE, but also other fluorescent contents of skin are measured with this method; (ii) some AGE are not fluorescent; and (iii) the measurement could be influenced by skin abnormalities.[32]

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REFERENCES

1. Rayner HC, Pisoni RL, Bommer J *et al.* Mortality and hospitalization in haemodialysis patients in five European countries: results from the dialysis outcomes and practice patterns study (DOPPS). *Nephrol. Dial. Transplant.* 2004; **19**: 108–20.
2. Ganesh SK, Hulbert-Shearon T, Port FK, Eagle K, Stack AG. Mortality differences by dialysis modality among incident ESRD patients with and without coronary artery disease. *J. Am. Soc. Nephrol.* 2003; **14**: 415–24.
3. Bevc S, Sabic S, Hojs R. Atherosclerosis in hemodialysis patients – the role of microinflammation. *Ren. Fail.* 2008; **30**: 1012–16.
4. Zimmermann J, Herrlinger S, Pruy A, Metzger T, Wanner C. Inflammation enhances cardiovascular risk and mortality in hemodialysis patients. *Kidney Int.* 1999; **55**: 648–58.
5. Piroddi M, Depunzio I, Calabrese V *et al.* Oxidatively-modified and glycated proteins as candidate pro-inflammatory toxins in uremia and dialysis patients. *Amino Acids* 2007; **32**: 573–92.
6. Suliman ME, Heimburger O, Barany P *et al.* Plasma pentosidine is associated with inflammation and malnutrition in end-stage renal disease patients starting on dialysis therapy. *J. Am. Soc. Nephrol.* 2003; **14**: 1614–22.
7. Yu TM, Chen YH, Hsu JY *et al.* Systemic inflammation is associated with pulmonary hypertension in patients undergoing haemodialysis. *Nephrol. Dial. Transplant.* 2009; **24**: 1946–51.
8. Krane V, Winkler K, Drechsler C, Lilienthal J, Marz W, Wanner C. Association of LDL cholesterol and inflammation with cardiovascular events and mortality in hemodialysis patients with type 2 diabetes mellitus. *Am. J. Kidney Dis.* 2009; **54**: 902–11.
9. Cai W, Zhu L, Chen X, Uribarri J, Peppa M. Association of advanced glycoxidation end products and inflammation markers with thrombosis of arteriovenous grafts in hemodialysis patients. *Am. J. Nephrol.* 2006; **26**: 181–5.
10. Brownlee M, Cerami A, Vlassara H. Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. *N. Engl. J. Med.* 1988; **318**: 1315–21.
11. Grandhee SK, Monnier VM. Mechanism of formation of the Maillard protein cross-link pentosidine. Glucose, fructose, and ascorbate as pentosidine precursors. *J. Biol. Chem.* 1991; **266**: 11649–53.
12. Dyer DG, Blackledge JA, Thorpe SR, Baynes JW. Formation of pentosidine during nonenzymatic browning of proteins by glucose. Identification of glucose and other carbohydrates as possible precursors of pentosidine *in vivo*. *J. Biol. Chem.* 1991; **266**: 11654–60.
13. Makita Z, Radoff S, Rayfield EJ *et al.* Advanced glycosylation end products in patients with diabetic nephropathy. *N. Engl. J. Med.* 1991; **325**: 836–42.
14. Vlassara H. Recent progress in advanced glycation end products and diabetic complications. *Diabetes* 1997; **46** (Suppl 2): S19–25.
15. Nakamura T, Sato E, Fujiwara N *et al.* Positive association of serum levels of advanced glycation end products and high mobility group box-1 with asymmetric dimethylarginine in nondiabetic chronic kidney disease patients. *Metabolism* 2009; **58**: 1624–8.
16. Yamagishi S. Advanced glycation end products and receptor-oxidative stress system in diabetic vascular complications. *Ther. Apher. Dial.* 2009; **13**: 534–9.
17. Kilhovd BK, Juutilainen A, Lehto S *et al.* High serum levels of advanced glycation end products predict increased coronary heart disease mortality in nondiabetic women but not in nondiabetic men: a population-based 18-year follow-up study. *Arterioscler. Thromb. Vasc. Biol.* 2005; **25**: 815–20.
18. Kilhovd BK, Juutilainen A, Lehto S *et al.* Increased serum levels of advanced glycation endproducts predict total, cardiovascular and coronary mortality in women with type 2 diabetes: a population-based 18 year follow-up study. *Diabetologia* 2007; **50**: 1409–17.
19. Lutgers HL, Graaff R, Links TP *et al.* Skin autofluorescence as a noninvasive marker of vascular damage in patients with type 2 diabetes. *Diabetes Care* 2006; **29**: 2654–9.
20. Meerwaldt R, Hartog JW, Graaff R *et al.* Skin autofluorescence, a measure of cumulative metabolic stress and advanced glycation end products, predicts mortality in hemodialysis patients. *J. Am. Soc. Nephrol.* 2005; **16**: 3687–93.
21. Yamada S, Inaba M, Kurajoh M *et al.* Utility of serum tartrate-resistant acid phosphatase (TRACP5b) as a bone resorption marker in patients with chronic kidney disease: independence from renal dysfunction. *Clin. Endocrinol. (Oxf.)* 2008; **69**: 189–96.
22. Daugirdas JT. Linear estimates of variable-volume, single-pool Kt/V: an analysis of error. *Am. J. Kidney Dis.* 1993; **22**: 267–70.
23. Gosling RG, King DH. Arterial assessment by Doppler-shift ultrasound. *Proc. R. Soc. Med.* 1974; **67**: 447–9.
24. Matsumoto T, Tsurumoto T, Baba H *et al.* Measurement of advanced glycation endproducts in skin of patients with rheumatoid arthritis, osteoarthritis, and dialysis-related spondyloarthropathy using non-invasive methods. *Rheumatol. Int.* 2007; **28**: 157–60.
25. Arsov S, Graaff R, Morariu AM *et al.* Does hepatitis C increase the accumulation of advanced glycation end products in haemodialysis patients? *Nephrol. Dial. Transplant.* 2009.
26. Yoshida T, Yamagishi S, Nakamura K *et al.* Telmisartan inhibits AGE-induced C-reactive protein production through downregulation of the receptor for AGE via peroxisome proliferator-activated receptor-gamma activation. *Diabetologia* 2006; **49**: 3094–9.
27. Tan KC, Chow WS, Tam S, Bucala R, Betteridge J. Association between acute-phase reactants and advanced glycation end products in type 2 diabetes. *Diabetes Care* 2004; **27**: 223–8.
28. Nakatou T, Nakata K, Nakamura A, Itoshima T. Carotid haemodynamic parameters as risk factors for cerebral infarction in Type 2 diabetic patients. *Diabet. Med.* 2004; **21**: 223–9.
29. Mulder DJ, van Haelst PL, Gross S *et al.* Skin autofluorescence is elevated in patients with stable coronary artery disease and is associated with serum levels of neopterin and the soluble receptor for advanced glycation end products. *Atherosclerosis* 2008; **197**: 217–23.
30. Stenvinkel P, Heimburger O, Paultre F *et al.* Strong association between malnutrition, inflammation, and atherosclerosis in chronic renal failure. *Kidney Int.* 1999; **55**: 1899–911.
31. Eleftheriadis T, Kartsios C, Antoniadi G *et al.* The impact of chronic inflammation on bone turnover in hemodialysis patients. *Ren. Fail.* 2008; **30**: 431–7.
32. Mulder DJ, Water TV, Lutgers HL *et al.* Skin autofluorescence, a novel marker for glycemic and oxidative stress-derived advanced glycation endproducts: an overview of current clinical studies, evidence, and limitations. *Diabetes Technol. Ther.* 2006; **8**: 523–35.

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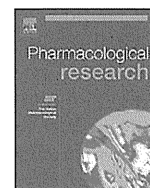

Pigment epithelium-derived factor (PEDF) inhibits proximal tubular cell injury in early diabetic nephropathy by suppressing advanced glycation end products (AGEs)-receptor (RAGE) axis

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ABSTRACT

Pigment epithelium-derived factor (PEDF) is a multifunctional glycoprotein with anti-angiogenic and anti-inflammatory properties, and it could block the development and progression of experimental diabetic retinopathy. However, a role for PEDF in early experimental diabetic nephropathy is not fully understood. Advanced glycation end products (AGEs) and their receptor (RAGE) axis stimulates oxidative stress generation and subsequently evokes inflammatory and fibrogenic reactions in renal tubular cells, thereby playing a role in diabetic nephropathy. Therefore, this study investigated whether PEDF could prevent AGE-elicited tubular cell injury in early diabetic nephropathy. Human proximal tubular cells were incubated with or without AGE-bovine serum albumin in the presence or absence of PEDF. Streptozotocin-induced diabetic rats were treated with or without intravenous injection of PEDF for 4 weeks. Gene expression was analyzed by quantitative real-time reverse transcription-polymerase chain reactions. Reactive oxygen species (ROS) was measured with dihydroethidium staining. PEDF or antibodies raised against RAGE inhibited the AGE-induced RAGE gene expression and subsequently reduced ROS generation, monocyte chemoattractant protein-1 (MCP-1) and transforming growth factor-β (TGF-β), fibronectin and type IV collagen mRNA levels in proximal tubular cells. RAGE gene expression, ROS generation and MCP-1 and TGF-β mRNA levels were significantly increased in diabetic kidney, which were suppressed by administration of PEDF. Our present data suggest that PEDF could play a protective role against tubular injury in diabetic nephropathy by attenuating the deleterious effects of AGEs via down-regulation of RAGE expression. Administration of PEDF may offer a promising strategy for halting the development of diabetic nephropathy.

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1. Introduction

Diabetic nephropathy is a leading cause of end-stage renal disease, and accounts for disabilities and the high mortality rates in patients with diabetes [1]. According to the World Health Organization, it is expected that the number of patients with diabetes will rise to 370 million by 2030 in the world [2]. It has also been reported that about 25–40% of type 1 or type 2 diabetic patients develop diabetic nephropathy within 20–25 year of the onset of diabetes [3]. Diabetic nephropathy is characterized by functional and structural changes in the glomerulus, such as glomerular hyperfiltration, thickening of glomerular basement membranes and an expansion of extracellular matrix in mesangial areas [4]. However, it has recently been recognized that changes within tubulointerstitium are more important than glomerulopathy in terms of renal prognosis in diabetic nephropathy [5,6].

Reducing sugars can react non-enzymatically with the amino groups of proteins to form Amadori products [7–9]. These early glycation products undergo further complex reaction, such as rearrangement, dehydration and condensation, to become

Abbreviations: AGEs, advanced glycation end products; RAGE, receptor for AGEs; PEDF, pigment epithelium-derived factor; BSA, bovine serum albumin; Ab, antibody; RAGE-Ab, Ab raised against human RAGE; RT-PCR, real-time reverse transcription-polymerase chain reactions; DHE, dihydroethidium; DM, diabetes mellitus; MCP-1, monocyte chemoattractant protein-1; TGF-β, transforming growth factor-β; SD, standard deviation; ROS, reactive oxygen species; PPARγ, peroxisome proliferator-activated receptor-γ.

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irreversibly cross-linked, heterogeneous fluorescent derivatives, termed advanced glycation end products (AGEs) [7–9]. The formation and accumulation of AGEs have been known to progress under diabetic conditions [7–9]. There is a growing body of evidence that AGEs and RAGE (receptor for AGEs) interaction stimulates oxidative stress generation and subsequently evokes inflammatory and fibrogenic reactions, thereby causing progressive alteration in renal architecture and loss of renal function in diabetes [10–15]. Therefore, the AGE–RAGE-induced oxidative stress in the kidney may be a therapeutic target for diabetic nephropathy.

Pigment epithelium-derived factor (PEDF), a glycoprotein that belongs to the superfamily of serine protease inhibitors, was first identified as a retinal pigment epithelium-derived protein with neuronal differentiating activity in human retinoblastoma cells [16,17]. Recently, PEDF has been shown to have potent anti-angiogenic activity in cell culture and animal models [18,19]. Further, we, along with others, have found that PEDF not only blocks AGE-induced endothelial cell damage, angiogenesis, and platelet activation *in vitro* through its anti-oxidative properties, but also reduces vascular inflammation and hyperpermeability in experimental diabetic retinopathy [20–31]. However, a role of PEDF in early diabetic nephropathy is not fully understood. Therefore, this study investigated whether PEDF could prevent AGE-elicited tubular cell injury in early phase of experimental diabetic nephropathy.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA) (essentially fatty acid free and essentially globulin free, lyophilized powder) was purchased from Sigma (St. Louis, MO, USA). D-Glyceraldehyde from Nakalai Tesque (Kyoto, Japan).

2.2. Preparation of AGE-BSA

AGE-BSA was prepared as described previously [32]. In brief, BSA (25 mg/ml) was incubated under sterile conditions with 0.1 M glyceraldehyde in 0.2 M $NaPO_4$ buffer (pH 7.4) for 7 days. Then unincorporated sugars were removed by PD-10 column chromatography and dialysis against phosphate-buffered saline. Control non-glycated BSA was incubated in the same conditions except for the absence of reducing sugars. Preparations were tested for endotoxin using Endospecy ES-20S system (Seikagaku Co., Tokyo, Japan); no endotoxin was detectable. The extent of chemical modification was determined as described with 2,4,6-trinitrobenzenesulfonic acid as a difference in lysine residues of modified and unmodified protein preparations [32]. The extent of lysine modification (%) of modified BSA preparations was 65% for AGE-BSA.

2.3. Preparation of PEDF proteins

PEDF proteins were purified as described previously [33]. SDS-PAGE analysis of purified PEDF proteins revealed a single band with a molecular weight of about 50 kDa, which showed positive reactivity with monoclonal antibody (Ab) raised against human PEDF (Transgenic, Kumamoto, Japan).

2.4. Preparation of RAGE Ab

Ab raised against human RAGE (RAGE-Ab) for neutralizing assays, which recognizes the amino acid residues 167–180 of human RAGE protein, was prepared as described previously [34].

2.5. Cells

Proximal tubular epithelial cells from human kidney were maintained in basal medium supplemented with 5% fetal bovine serum, 0.5 μg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, 0.5 μg/ml epinephrine, 6.5 ng/ml triiodo-L-thyronine, 10 μg/ml transferrin, 5 μg/ml insulin, and GA-1000 according to the supplier's instructions (Clonetics Corp., San Diego, CA, USA) [35]. Cells at 3–5 passages were used for the experiments. AGE treatments were carried out in a serum-free basal medium containing 10 μg/ml transferrin and GA-1000. Tubular cells were treated with 100 μg/ml AGE-BSA or non-glycated BSA in the presence or absence of 10 nM PEDF or 5 μg/ml Ab raised against RAGE for 4 h. Then real-time reverse transcription-polymerase chain reactions (RT-PCR) and dihydroethidium (DHE) staining were performed.

2.6. Induction of experimental diabetes

Six wk-old male Wister rats received single 60 mg/kg intraperitoneal injection of streptozotocin (Sigma) in 10 mmol/l citrate buffer (pH 4.5). Non-diabetic control rats received citrate buffer alone. Animals with blood glucose levels greater than 250 mg/dl 48 h later were considered diabetic. Wister rats were divided into 4 groups (Control, PEDF, diabetes mellitus (DM) and DM + PEDF). Diabetic or non-diabetic control rats were injected intravenously with PEDF (5 μg PEDF/100 g body weight) or vehicle 3 times a week for up to 4 weeks. Diabetic rats were maintained with subcutaneous injections of 2 U insulin (2 times/week) (Humalin N, Eli Lilly, Indianapolis, IN, USA). On the day of 4 weeks, body weight was measured. Then the rats were killed and kidneys were removed. Blood chemistries, including serum creatinine were measured with standard enzymatic methods as described previously [36]. All animal procedures were conducted according to the guidelines provided by the Kurume University Institutional Animal Care and Use Committee under an approved protocol.

2.7. RT-PCR

Total RNAs were extracted from cultured proximal tubular cells or rat kidneys with RNAqueous-4PCR kit (Ambion Inc., Austin, TX, USA) according to the manufacturer's instructions. Quantitative real-time RT-PCR was performed using Assay-on-Demand and TaqMan 5 fluorogenic nuclease chemistry (Applied Biosystems, Foster city, CA, USA) according to the suplier's recommendation. IDs of primers for rat RAGE, monocyte chemoattractant protein-1 (MCP-1), transforming growth factor-β (TGF-β), and β-actin gene were Rn00584249_m1, Rn00580555_m1, Rn99999016_m1 and Rn00667869_m1, respectively. IDs of primers for human RAGE, MCP-1, TGF-β, fibronectin, type IV collagen and β-actin gene were Hs00153957_m1, Hs00234140_m1, Hs00171257_m1, Hs01549940_m1, Hs00266237_m1 and Hs99999903_m1, respectively.

2.8. DHE staining

Tubular cells or frozen kidney sections were incubated with phenol red free Dulbecco's Modified Eagle Medium containing 3 μM DHE (Molecular Probes Inc., Eugene, OR, USA) as described previously [37]. After 15 min, images were obtained by a laser-scanning confocal microscope. Intensity of DHE staining in 5 different field of each sample was analyzed by microcomputer-assisted image J.

2.9. Flow cytometric analysis of apoptotic cells

Apoptotic cells were measured by flow cytometry as described previously [38]. In brief, tubular cells were treated with 100 μg/ml

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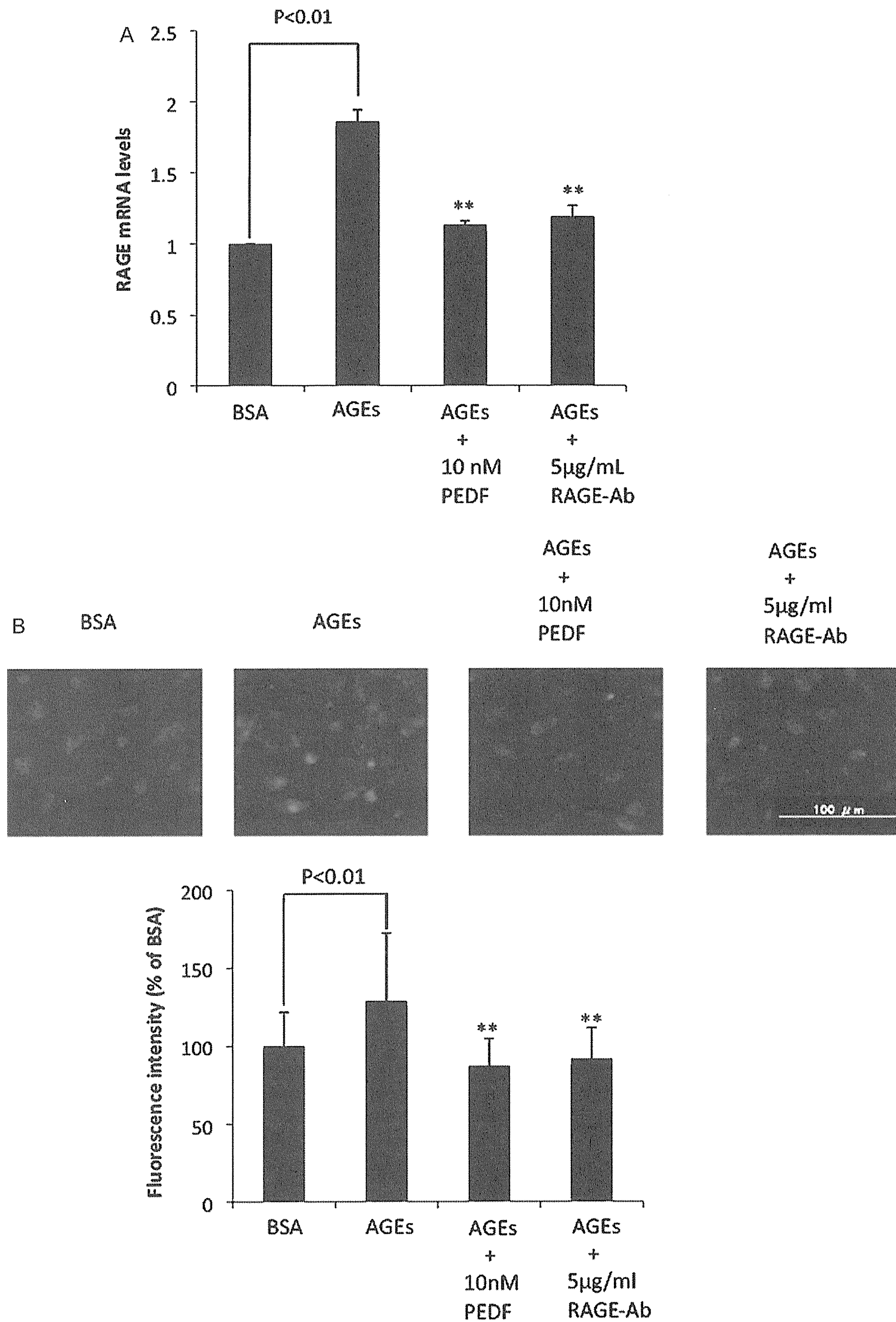

Fig. 1. Effects of PEDF on RAGE gene expression (A) and ROS generation (B) in tubular cells. Cells were treated with 100 μg/ml AGE-BSA or non-glycated BSA in the presence or absence of 10 nM PEDF or 5 μg/ml RAGE-Ab for 4 h. (A) Total RNAs were transcribed and amplified by real-time PCR. Data were normalized by the intensity of β-actin mRNA-derived signals and then related to the value obtained with non-glycated BSA. (B) Cells were incubated with DHE. Upper panel shows typical microphotographs of the cells. Lower panel shows quantitative data of ROS generation evaluated by fluorescent intensity. Data are presented as mean ± standard error. $^{**}p < 0.01$ compared to the value with AGEs alone. $N = 4–5$ per group.

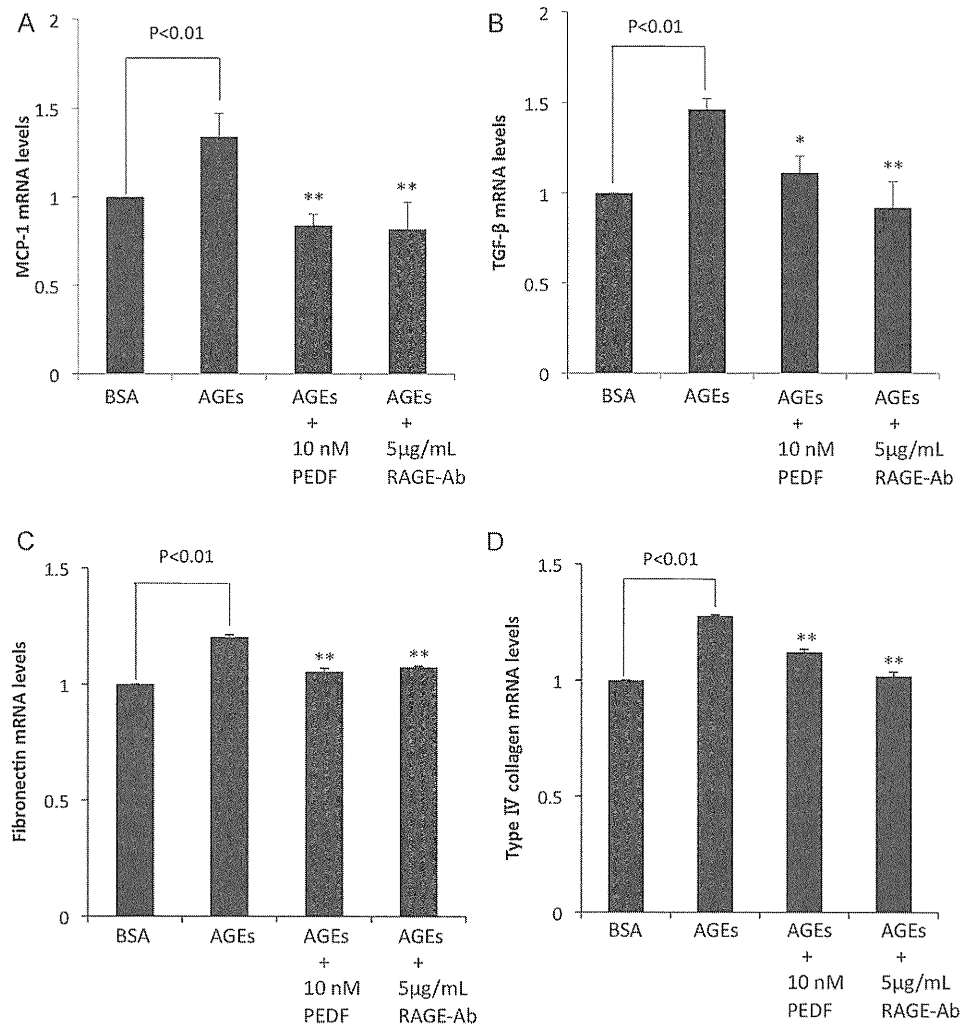

Fig. 2. Effects of PEDF on MCP-1 (A), TGF-β (B), fibronectin (C) and type IV collagen (D) gene expression in tubular cells. Cells were treated with 100 μg/ml AGE-BSA or non-glycated BSA in the presence or absence of 10 nM PEDF or 5 μg/ml RAGE-Ab for 4 h. Total RNAs were transcribed and amplified by real-time PCR. Data were normalized by the intensity of β-actin mRNA-derived signals and then related to the value obtained with non-glycated BSA. * and ** $p < 0.05$ and $p < 0.01$ compared to the value with AGEs alone, respectively. $N = 3–4$ per group.

AGE-BSA or non-glycated BSA in the presence or absence of 10 nM PEDF for 4 h. Then the cells were incubated with fluorescein isothiocyanate-conjugated annexin V and then analyzed by flow cytometry according to the supplier's recommendations (Clontech Laboratories, Inc., Mountain View, CA, USA). For every histogram, 10,000 tubular cells were counted to evaluate the percentage of apoptotic cells.

2.10. Statistics

Unless otherwise indicated, all values were presented as means ± standard deviation (SD). One-way ANOVA followed by the Scheffe *F* test was performed for statistical comparisons; $p < 0.05$ was considered significant.

3. Results

We first investigated whether PEDF could inhibit RAGE gene expression in tubular cells. Quantitative real-time RT-PCR analysis revealed that 100 μg/ml AGE-BSA up-regulated RAGE mRNA levels in tubular cells; compared with non-glycated BSA, 4-h treatment with AGE-BSA increased RAGE mRNA levels by about 1.7-fold (Fig. 1A). Ten nM PEDF or 5 μg/ml RAGE-Ab was found completely to inhibit the AGE-induced increase in RAGE mRNA levels in tubular cells (Fig. 1A). Since relatively high dose of AGEs (500 μg/ml AGEs) did not dramatically increase RAGE mRNA level in tubular cells (it only increased RAGE mRNA level to about 1.1-fold, data not shown), we chose the concentrations of 100 μg/ml of AGEs for the following experiments.

AGEs exert pleiotropic actions on various types of cells by inducing the generation of intracellular reactive oxygen species (ROS) through the interaction with cell surface receptor, RAGE [10–15]. So, we investigated whether PEDF could block the AGE-elicited ROS generation in tubular cells. As shown in Fig. 1B, AGE-BSA increased ROS generation in tubular cells, which was also completely blocked by the treatment with 10 nM PEDF or 5 μg/ml RAGE-Ab. We have previously confirmed that AGEs induce ROS generation in human proximal tubular cells, the same cells used in the present experiments with other method such as CM-H_2-DCF-DA [35].

We next examined whether PEDF could exert anti-inflammatory effects on AGE-exposed tubular cells. As shown in Fig. 2A–D, 10 nM PEDF or 5 μg/ml RAGE-Ab significantly inhibited the AGE-induced increase in MCP-1, TGF-β, fibronectin and type IV collagen mRNA

Table 1
Clinical variables at 4 weeks after each treatment.

	Control	PEDF	DM	DM + PEDF
Body weight (g)	337.8 ± 17	339 ± 45.8	248.9 ± 52.2*	238 ± 37.9
Glucose (mg/dl)	73.7 ± 10	108.4 ± 85.8	514.8 ± 78.7*	489 ± 96.7
Cholesterol (mg/dl)	63.8 ± 14.2	65.0 ± 6.2	167.8 ± 52.1*	154.8 ± 57.0
TG (mg/dl)	95.8 ± 27.3	87.5 ± 23.8	869.8 ± 743.4*	977.4 ± 416.6
HDL-cholesterol (mg/dl)	25.0 ± 4.2	25.5 ± 2.6	49.4 ± 9.1*	42.8 ± 9.3
Serum creatinine (mg/dl)	0.26 ± 0.03	0.29 ± 0.05	0.29 ± 0.05*	0.27 ± 0.09

* $p < 0.01$ compared with the values of control. $N = 6–7$ per group.

levels in tubular cells. Ten nM PEDF or 5 μg/ml RAGE-Ab alone did not affect ROS generation, RAGE, MCP-1 or TGF-β, fibronectin and type IV collagen gene expression in tubular cells (data not shown). About 100 μg/ml AGE-BSA significantly ($p < 0.05$) induced apoptotic cell death of tubular cells, but the effects of PEDF on apoptosis in AGE-exposed cells were modest, not significant; ratio of apoptosis in BSA-, AGEs, AGEs plus 10 nM PEDF-treated cells were 2.9 ± 1.6, 4.8 ± 1.7 and 3.7 ± 1.5%, respectively.

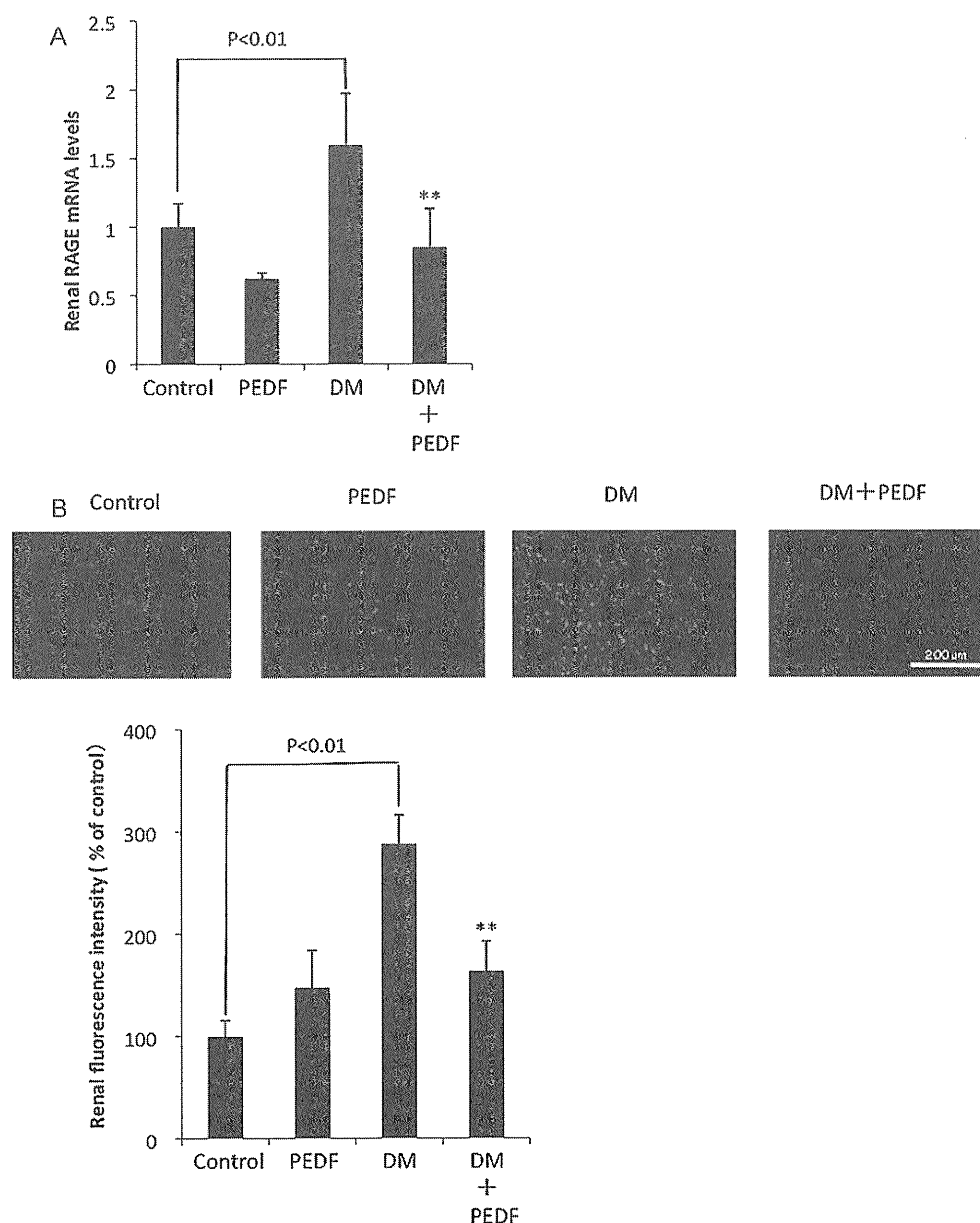

Fig. 3. Effects of PEDF administration on RAGE gene expression (A) and ROS generation (B) in the kidneys of each group. (A) Total RNAs extracted from the kidneys were transcribed and amplified by real-time PCR. Data were normalized by the intensity of β-actin mRNA-derived signals and then related to the value obtained with control rats (control). (B) Rat kidneys were embedded in OCT compound (Tissue-Tek, Sakura Finetechnical Co. Ltd., Tokyo, Japan) to freeze on dry ice and were cut into 10-μm sections. The frozen sections were incubated with DHE. Upper panel shows typical microphotographs. Lower panel shows quantitative data of ROS generation evaluated by fluorescent intensity. $^{**}p < 0.01$ compared to the value with diabetic rats (DM). $N = 4–8$ per group.

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Since tubular cells are major constituents of kidney, we next studied the effects of PEDF on experimental diabetic nephropathy. Although PEDF treatment did not affect body weight, blood glucose, lipids or serum creatinine level in diabetic animals, administration of PEDF significantly inhibited RAGE gene expression, ROS generation, MCP-1 and TGF-β mRNA levels in the kidney of diabetic rats (Table 1 and Figs. 3 and 4).

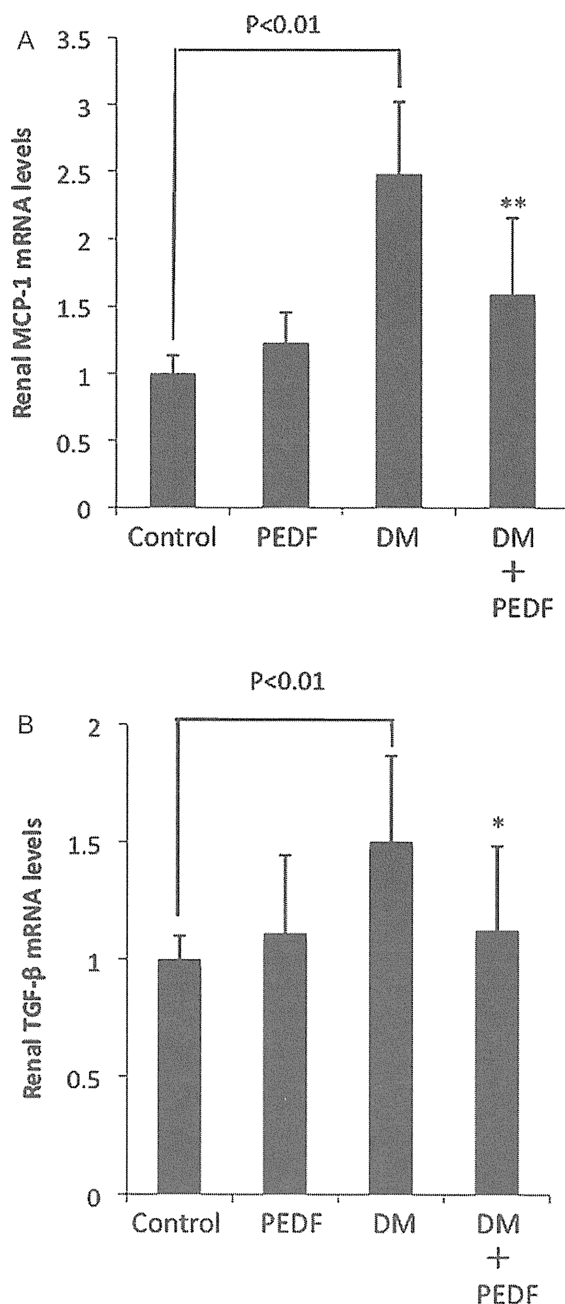

Fig. 4. Effects of PEDF administration on MCP-1 (A) and TGF-β (B) gene expression in the kidneys of each group. Total RNAs extracted from the kidneys were transcribed and amplified by real-time PCR. Data were normalized by the intensity of β-actin mRNA-derived signals and then related to the value obtained with control rats (Control). * and ** $p < 0.05$ and $p < 0.01$ compared to the value with diabetic rats (DM), respectively. $N = 4$–8 per group.

4. Discussion

In this study, we demonstrated for the first time that PEDF inhibited the AGE-induced RAGE gene expression and reduced ROS generation, inflammatory and fibrogenic gene expression (MCP-1, TGF-β, fibronectin and type IV collagen gene expression) in cultured human proximal tubular cells. We also found here that PEDF administration inhibited oxidative stress generation and RAGE, MCP-1 and TGF-β gene induction in diabetic kidneys, although it did not affect blood glucose or lipid level. MCP-1 and TGF-β gene expression in AGE-exposed tubular cells is suppressed by antioxidants [35,39,40], and TGF-β is one of the etiologic agents for fibrogenic reactions such as overexpression of fibronectin and type IV collagen in the diabetic kidney [6,34,35]. Given that RAGE is a major receptor for AGEs that mediates the downstream signaling and that ROS generation works as a second messenger of RAGE [10–15,39,40], the present study suggests that RAGE gene suppression in tubular cells would be a central mechanism by which PEDF inhibited inflammatory and fibrogenic reactions in early phase of experimental diabetic nephropathy. Chronic tubulointerstitial damage in the kidney, including tubulointerstitial inflammation and fibrosis, is more important than glomerulopathy in terms of renal prognosis in diabetic nephropathy [5,6]. Further, renal MCP-1 and TGF-β overexpressions are involved in tubulointerstitial injury [10,41,42]. Taken together, our present results suggest that blockade of RAGE by PEDF may play a protective role against tubular injury in diabetes by attenuating the deleterious effects of AGEs.

We have previously found that PEDF administration decreases RAGE mRNA levels in the eyes of diabetic rats, thereby protecting against the development and progression of diabetic retinopathy [26,27]. So, PEDF treatment could exert beneficial effects on both retinopathy and nephropathy by reducing RAGE expression in diabetes. In this study, we did not clarify the molecular mechanism of RAGE gene suppression by PEDF. However, peroxisome proliferator-activated receptor-γ (PPARγ) agonists have been recently reported to down-regulate AGE-induced RAGE gene expression in tubular cells and subsequently inhibit the AGE-induced intercellular adhesion molecule-1 and TGF-β expression, thus limiting the cells' susceptibility toward pro-inflammatory and pro-fibrogenic effects of AGEs [40]. Since PEDF activates PPARγ signaling in variety of cells [43,44], PEDF may reduce RAGE gene expression in diabetic kidney via PPARγ.

In this study, we examined the effects of PEDF at 10 nM in AGE-exposed tubular cells because this concentration of PEDF alone did not influence RAGE gene expression or ROS generation in tubular cells (data not shown). Since we have previously shown that 10 nM PEDF completely blocks the AGE actions on endothelial cells [28], this concentration of protein-unbound free form of PEDF could be efficient to suppress the AGE-signal *in vitro*. Recently, serum PEDF levels were found to be significantly higher in type 1 diabetic patients with microvascular complications compared to the patients without them (8.2 μg/ml vs. 5.3 μg/ml) [45]. These findings suggest that relatively high levels of PEDF are already present in the serum of diabetic patients with nephropathy. However, our recent study suggests that most of circulating PEDF in the serum may exist as a protein-bound form, but not protein-unbound free form [46]. Therefore, serum PEDF levels may not necessarily reflect its biological activity *in vivo*. This is one possible reason why small dose of free PEDF injected in this study (5 μg PEDF/100 g body weight) exerted beneficial effects on experimental diabetic nephropathy.

In vitro-modified AGEs were prepared by incubating BSA with glyceraldehyde for 1 week; this process produces relatively highly-modified proteins in comparison to those *in vivo*. However, it is unlikely that extensively-modified, unphysiologic AGEs that were formed under the *in vitro*-conditions may exert non-specific

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and toxic effects on tubular cell damage for the following reasons: we have previously found that immunological epitope of glyceraldehyde-modified AGEs was actually present in serum of diabetic patients and that the concentration (100 μg/ml) of *in vitro*-prepared AGEs used here were comparable with those of the *in vivo* diabetic situation [47,48]

5. Limitations

In this study, we evaluated the effect of PEDF administration on diabetic nephropathy using a 4-week type 1 diabetes model. A 4-week time point was selected specially to examine the effects of PEDF on very early phase of diabetic nephropathy before the onset of albuminuria and histological changes of the kidney, which usually takes place after at least 8 weeks of diabetes in this model [49]. So, it would be interesting to examine whether relatively long-term treatment of PEDF could improve other manifestations of diabetic nephropathy such as albuminuria and tubulointerstitial fibrosis in our model. With this regard, Wang et al. previously reported that infection of adenovirus expressing PEDF significantly decreased urinary albumin excretion and reduced extracellular matrix protein deposition in the kidney of other diabetic animal models [50,51]. These observations suggest that PEDF may have renoprotective properties in very early–early phases of diabetic nephropathy by blocking the AGE–RAGE axis. Anyway, since experimental animal model does not completely mimic human diabetic nephropathy, further study is needed to clarify whether PEDF treatment could be a therapeutic strategy for the treatment of early diabetic nephropathy in humans.

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References

[1] Yamagishi S, Fukami K, Ueda S, Okuda S. Molecular mechanisms of diabetic nephropathy and its therapeutic intervention. Curr Drug Targets 2007;8:952–9.
[2] Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 2004;27:1047–53.
[3] Remuzzi G, Schieppati A, Ruggenenti P. Clinical practice. Nephropathy in patients with type 2 diabetes. N Engl J Med 2002;346:1145–51.
[4] Yamagishi S, Matsui T. Advanced glycation end products, oxidative stress and diabetic nephropathy. Oxid Med Cell Longev 2010;3:1018.
[5] Taft JL, Nolan CJ, Yeung SP, Hewitson TD, Martin FI. Clinical and histological correlations of decline in renal function in diabetic patients with proteinuria. Diabetes 1994;43:1046–51.
[6] Ziyadeh FN, Goldfarb S. The renal tubulointerstitium in diabetes mellitus. Kidney Int 1991;39:464–75.
[7] Vlassara H, Bucala R. Recent progress in advanced glycation and diabetic vascular disease: role of advanced glycation end product receptors. Diabetes Suppl 1996;3:S65–6.
[8] Brownlee M, Cerami A, Vlassara A. Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. N Engl J Med 1998;318:1315–21.
[9] Rahbar S. Novel inhibitors of glycation and AGE formation. Cell Biochem Biophys 2007;48:147–57.
[10] Wendt TM, Tanji N, Guo J, Kislinger TR, Qu W, Lu Y, et al. RAGE drives the development of glomerulosclerosis and implicates podocyte activation in the pathogenesis of diabetic nephropathy. Am J Pathol 2003;162:1123–37.
[11] Yamamoto Y, Kato I, Doi T, Yonekura H, Ohashi S, Takeuchi M, et al. Development and prevention of advanced diabetic nephropathy in RAGE-overexpressing mice. J Clin Invest 2001;108:261–8.
[12] Yamagishi S, Imaizumi T. Diabetic vascular complications: pathophysiology, biochemical basis and potential therapeutic strategy. Curr Pharm Des 2005;11:2279–99.
[13] Yamagishi S, Nakamura K, Matsui T, Ueda S, Fukami K, Okuda S. Agents that block advanced glycation end product (AGE)-RAGE (receptor for AGEs)-oxidative stress system: a novel therapeutic strategy for diabetic vascular complications. Expert Opin Invest Drugs 2008;17:983–96.
[14] Yamagishi S, Nakamura K, Matsui T, Noda Y, Imaizumi T. Receptor for advanced glycation end products (RAGE): a novel therapeutic target for diabetic vascular complication. Curr Pharm Des 2008;14:487–95.
[15] Fukami K, Yamagishi S, Ueda S, Okuda S. Role of AGEs in diabetic nephropathy. Curr Pharm Des 2008;14:946–52.
[16] Tombran-Tink J, Chader CG, Johnson LV. PEDF: pigment epithelium-derived factor with potent neuronal differentiative activity. Exp Eye Res 1991;53:411–4.
[17] Tombran-Tink J, Barnstable CJ. PEDF: a multifaceted neurotrophic factor. Nat Rev Neurosci 2003;4:628–36.
[18] Dawson DW, Volpert OV, Gillis P, Crawford SE, Xu HJ, Benedict W, et al. Pigment epithelium-derived factor: a potent inhibitor of angiogenesis. Science 1999;285:245–8.
[19] Duh EJ, Yang HS, Suzuma I, Miyagi M, Youngman E, Mori K, et al. Pigment epithelium-derived factor suppresses ischemia-induced retinal neovascularization and VEGF-induced migration and growth. Invest Ophthalmol Vis Sci 2002;43:821–9.
[20] Inagaki Y, Yamagishi S, Okamoto T, Takeuchi M, Amano S. Pigment epithelium-derived factor prevents advanced glycation end products-induced monocyte chemoattractant protein-1 production in microvascular endothelial cells by suppressing intracellular reactive oxygen species generation. Diabetologia 2003;46:284–7.
[21] Yamagishi S, Ueda S, Matsui T, Nakamura K, Imaizumi T, Takeuchi M, et al. Pigment epithelium-derived factor (PEDF) prevents advanced glycation end products (AGEs)-elicited endothelial nitric oxide synthase (eNOS) reduction through its anti-oxidative properties. Protein Pept Lett 2007;14:832–5.
[22] Sheikpranbabu S, Haribalaganesh R, Lee KJ, Gurunathan S. Pigment epithelium-derived factor inhibits advanced glycation end products-induced retinal vascular permeability. Biochimie 2010;92:1040–51.
[23] Zhang SX, Wang JJ, Gao G, Shao C, Mott R, Ma JX. Pigment epithelium-derived factor (PEDF) is an endogenous antiinflammatory factor. FASEB J 2006;20:323–5.
[24] Yamagishi S, Matsui T, Takenaka K, Nakamura K, Takeuchi M, Inoue H. Pigment epithelium-derived factor prevents platelet activation and aggregation in diabetic rats by blocking deleterious effects of advanced glycation end products (AGEs). Diabetes Metab Res Rev 2009;25:266–71.
[25] Yamagishi S, Matsui T, Nakamura K, Yoshida T, Takeuchi M, Imaizumi T. Pigment epithelium-derived factor (PEDF) blocks advanced glycation end product (AGE)-induced angiogenesis in vitro. Horm Metab Res 2007;39:233–5.
[26] Yamagishi S, Matsui T, Nakamura K, Yoshida T, Takeuchi M, Inoue H, et al. Pigment-epithelium-derived factor suppresses expression of receptor for advanced glycation end products in the eye of diabetic rats. Ophthalmic Res 2007;39:92–7.
[27] Yamagishi S, Matsui T, Nakamura K, Takeuchi M, Imaizumi T. Pigment epithelium-derived factor (PEDF) prevents diabetes- or advanced glycation end products (AGE)-elicited retinal leukostasis. Microvasc Res 2006;72: 86–90.
[28] Yamagishi S, Nakamura K, Matsui T, Inagaki Y, Takenaka K, Jinnouchi Y, et al. Pigment epithelium-derived factor inhibits advanced glycation end product-induced retinal vascular hyperpermeability by blocking reactive oxygen species-mediated vascular endothelial growth factor expression. J Biol Chem 2006;281:20213–20.
[29] Yoshida Y, Yamagishi S, Matsui T, Jinnouchi Y, Fukami K, Imaizumi T, et al. Protective role of pigment epithelium-derived factor (PEDF) in early phase of experimental diabetic retinopathy. Diabetes Metab Res Rev 2009;25:678–86.
[30] Yamagishi S, Matsui T, Nakamura K, Ueda S, Noda Y, Imaizumi T. Pigment epithelium-derived factor (PEDF): its potential therapeutic implication in diabetic vascular complications. Curr Drug Targets 2008;9:1025–9.
[31] Yamagishi S, Matsui T. Anti-atherothrombogenic properties of PEDF. Curr Mol Med 2010;10:284–91.
[32] Yamagishi S, Inagaki Y, Okamoto T, Amano S, Koga K, Takeuchi M, et al. Advanced glycation end product-induced apoptosis and overexpression of vascular endothelial growth factor and monocyte chemoattractant protein-1 in human-cultured mesangial cells. J Biol Chem 2002;277:20309–15.
[33] Yamagishi S, Inagaki Y, Amano S, Okamoto T, Takeuchi M, Makita Z. Pigment epithelium-derived factor protects cultured retinal pericytes from advanced glycation end product-induced injury through its antioxidative properties. Biochem Biophys Res Commun 2002;296:877–82.
[34] Fukami K, Ueda S, Yamagishi S, Kato S, Inagaki Y, Takeuchi M, et al. AGEs activate mesangial TGF-beta-Smad signaling via an angiotensin II type I receptor interaction. Kidney Int 2004;66:2137–47.
[35] Yamagishi S, Inagaki Y, Okamoto T, Amano S, Koga K, Takeuchi M. Advanced glycation end products inhibit de novo protein synthesis and induce TGF-beta overexpression in proximal tubular cells. Kidney Int 2003;63:464–73.
[36] Yamagishi S, Matsui T, Adachi H, Takeuchi M. Positive association of circulating levels of advanced glycation end products (AGEs) with pigment epithelium-derived factor (PEDF) in a general population. Pharmacol Res 2010;61: 103–7.
[37] Nakamura K, Yamagishi S, Matsui T, Yoshida T, Takenaka K, Jinnouchi Y, et al. Pigment epithelium-derived factor inhibits neointimal hyperplasia after vascular injury by blocking NADPH oxidase-mediated reactive oxygen species generation. Am J Pathol 2007;170:2159–70.
[38] Takenaka K, Yamagishi S, Matsui T, Nakamura K, Jinnouchi Y, Yoshida Y, et al. Pigment epithelium-derived factor (PEDF) administration inhibits occlusive thrombus formation in rats: a possible participation of reduced intraplatelet PEDF in thrombosis of acute coronary syndromes. Atherosclerosis 2008;197:25–33.

[39] Matsui T, Yamagishi S, Takeuchi M, Ueda S, Fukami K, Okuda S. Irbesartan inhibits advanced glycation end product (AGE)-induced proximal tubular cell injury in vitro by suppressing receptor for AGEs (RAGE) expression. Pharmacol Res 2010;61:34–9.
[40] Matsui T, Yamagishi S, Takeuchi M, Ueda S, Fukami K, Okuda S. Nifedipine inhibits advanced glycation end products (AGEs) and their receptor (RAGE) interaction-mediated proximal tubular cell injury via peroxisome proliferator-activated receptor-gamma activation. Biochem Biophys Res Commun 2010;398:326–30.
[41] Tesch GH. MCP-1/CCL2: a new diagnostic marker and therapeutic target for progressive renal injury in diabetic nephropathy. Am J Physiol Renal Physiol 2008;294:F697–701.
[42] Cooper ME. Interaction of metabolic and haemodynamic factors in mediating experimental diabetic nephropathy. Diabetologia 2001;44:1957–72.
[43] Ho TC, Chen SL, Yang YC, Liao CL, Cheng HC, Tsao YP. PEDF induces p53-mediated apoptosis through PPAR gamma signaling in human umbilical vein endothelial cells. Cardiovasc Res 2007;76:213–23.
[44] Ho TC, Yang YC, Chen SL, Kuo PC, Sytwu HK, Cheng HC, et al. Pigment epithelium-derived factor induces THP-1 macrophage apoptosis and necrosis by the induction of the peroxisome proliferator-activated receptor gamma. Mol Immunol 2008;45:898–909.
[45] Jenkins AJ, Zhang SX, Rowley KG, Karschimkus CS, Nelson CL, Chung JS, et al. Increased serum pigment epithelium-derived factor is associated with microvascular complications, vascular stiffness and inflammation in Type 1 diabetes. Diabet Med 2007;24:1345–51.
[46] Yamagishi S, Adachi H, Abe A, Yashiro T, Enomoto M, Furuki K, et al. Elevated serum levels of pigment epithelium-derived factor in the metabolic syndrome. J Clin Endocrinol Metab 2006;91:2447–50.
[47] Takeuchi M, Makita Z, Yanagisawa K, Kameda K, Koike T. Detection of noncarboxymethyllysine and carboxymethyllysine advanced glycation end products (AGE) in serum of diabetic patients. Mol Med 1999;5:393–405.
[48] Miura J, Yamagishi S, Uchigata Y, Takeuchi M, Yamamoto H, Makita Z, et al. Serum levels of non-carboxymethyllysine advanced glycation endproducts are correlated to severity of microvascular complications in patients with Type 1 diabetes. J Diabetes Compl 2003;17:16–21.
[49] Thomas MC, Tikellis C, Kantharidis P, Burns WC, Cooper ME, Forbes JM. The role of advanced glycation in reduced organic cation transport associated with experimental diabetes. J Pharmacol Exp Therap 2004;311:456–66.
[50] Wang JJ, Zhang SX, Mott R, Knapp RR, Cao W, Lau K, et al. Salutary effect of pigment epithelium-derived factor in diabetic nephropathy: evidence for antifibrogenic activities. Diabetes 2006;55:1678–85.
[51] Wang JJ, Zhang SX, Mott R, Chen Y, Knapp RR, Cao W, et al. Anti-inflammatory effects of pigment epithelium-derived factor in diabetic nephropathy. Am J Physiol Renal Physiol 2008;294:F1166–73.

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Research Article

High Glucose Increases Metallothionein Expression in Renal Proximal Tubular Epithelial Cells

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Metallothionein (MT) is an intracellular metal-binding, cysteine-rich protein, and is a potent antioxidant that protects cells and tissues from oxidative stress. Although the major isoforms MT-1 and -2 (MT-1/-2) are highly inducible in many tissues, the distribution and role of MT-1/-2 in diabetic nephropathy are poorly understood. In this study, diabetes was induced in adult male rats by streptozotocin, and renal tissues were stained with antibodies for MT-1/-2. MT-1/-2 expression was also evaluated in mProx24 cells, a mouse renal proximal tubular epithelial cell line, stimulated with high glucose medium and pretreated with the antioxidant vitamin E. MT-1/-2 expression was gradually and dramatically increased, mainly in the proximal tubular epithelial cells and to a lesser extent in the podocytes in diabetic rats, but was hardly observed in control rats. MT-1/-2 expression was also increased by high glucose stimulation in mProx24 cells. Because the induction of MT was suppressed by pretreatment with vitamin E, the expression of MT-1/-2 is induced, at least in part, by high glucose-induced oxidative stress. These observations suggest that MT-1/-2 is induced in renal proximal tubular epithelial cells as an antioxidant to protect the kidney from oxidative stress, and may offer a novel therapeutic target against diabetic nephropathy.

1. Introduction

Diabetic nephropathy is a leading cause of end-stage renal disease, and many mechanisms have been proposed to explain the pathogenesis of renal injury in diabetes [1]. Recent studies have shown that hyperglycemia may induce oxidative stress by increasing reactive oxygen species (ROS) generation in the diabetic kidney [2–4] and that overexpression of the antioxidant superoxide dismutase 1 attenuated diabetic nephropathy in streptozotocin (STZ)-induced and *db*/*db* diabetic mice [5, 6]. Therefore, ROS could be an important mediator of diabetic nephropathy, and protection from ROS might offer a valuable therapeutic strategy to treat diabetic nephropathy.

Metallothionein (MT) is an intracellular metal-binding protein with a low-molecular mass (6-7 kDa) and a high cysteine content (20 of 61-62 amino acids). Its major isoforms, MT-1 and -2 (MT-1/-2), are widely distributed

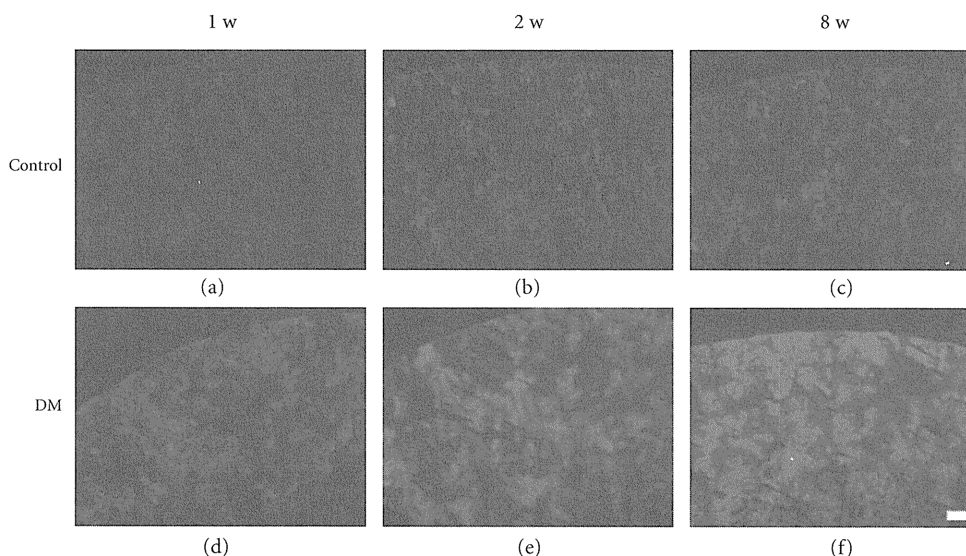


Figure 1: MT-1/-2 expression in the kidney. Diabetes was induced by injection of streptozotocin, and kidneys were obtained at 1 (a and d), 2 (b and e), or 8 (c and f) weeks after inducing diabetes. Immunofluorescent staining was performed as described in *Materials and Methods*. MT was strongly expressed in the renal cortex of diabetic rats (d, e, f) and hardly expressed in control rats (a, b, c). The expression of MT-1/-2 was greater at week 8 than at weeks 1 and 2 after diabetes induction. Scale bar: 100 μm.

throughout the body [7, 8]. Since MT-1/-2 expression is significantly upregulated by overload of essential trace metals (e.g., Zn and Cu), it plays an important role in heavy metal detoxification and essential metal homeostasis [9, 10]. In addition, MTs have been shown to act as nonspecific free radical scavengers [11, 12], suggesting that they exert antioxidant activities in various diseases, including diabetic nephropathy.

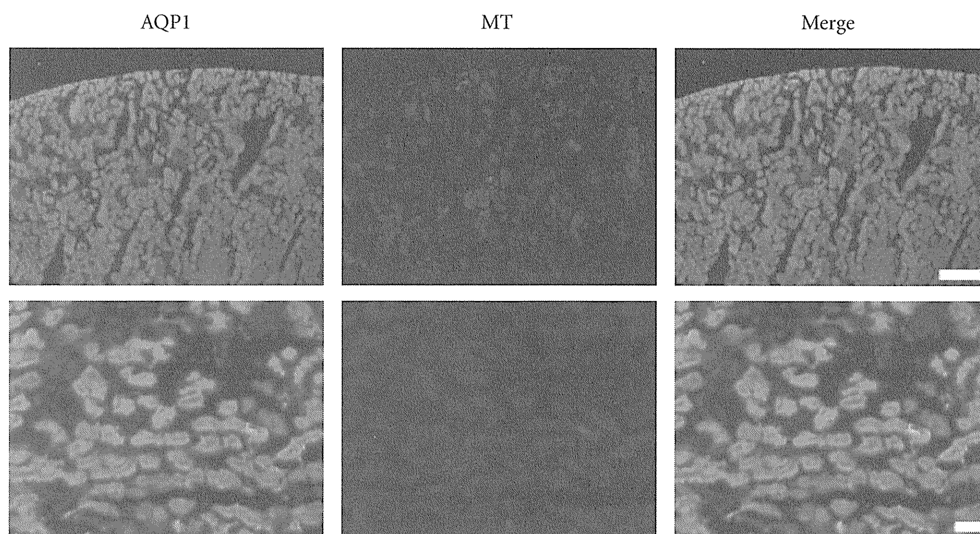
We and other investigators have demonstrated that MTs have neuroprotective effects in mouse models of Parkinson's disease [13–15]. In contrast, the role of MTs in the pathogenesis of diabetic nephropathy is poorly understood. Several studies reported that renal expression of MT is increased in STZ-induced diabetic rats [16], diabetic BB rats [17], and *ob/ob* diabetic mice [18]. However, the distribution of MTs in the diabetic kidney and the mechanisms by which MTs are induced in diabetes are poorly understood. Therefore, in the present study, we investigated the expression and localization of MT-1/-2 during the development of diabetic nephropathy and explored the mechanism by which MT-1/-2 expression was induced by high glucose in the kidney.

2. Materials and Methods

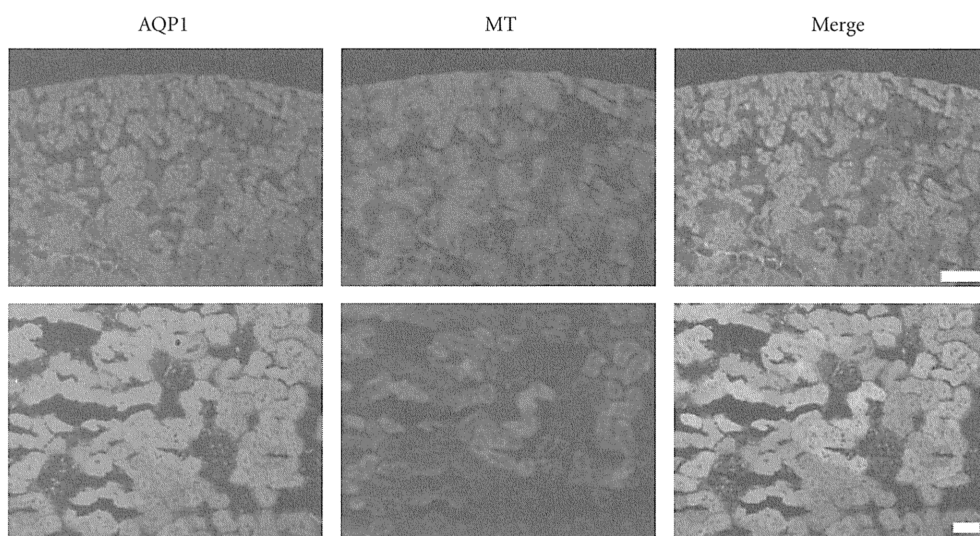
2.1. Experimental Protocol. Male Sprague Dawley rats were purchased from Charles River (Yokohama, Japan). Five-week-old rats were divided into two groups: (1) nondiabetic control rats (control; $n = 6$) and (2) STZ-induced diabetic rats (DM; $n = 6$). Diabetes was induced by peritoneal injection of 200 mg/kg STZ (Sigma-Aldrich Corp., MO) in citrate buffer (pH 4.5). Blood glucose was measured by the glucose oxidase method at 3 days after STZ injection and only rats with blood glucose concentrations >16 mmol/L were used in the study. All rats had free access to standard diet 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 the Japanese Government Notification on Feeding and Safekeeping of Animals (No. 6). Rats were sacrificed at 1, 2, or 8 weeks after inducing diabetes. We measured body weight, hemoglobin A1c (HbA1c), and 24-h urinary albumin excretion (UAE) at 1, 2, and 8 weeks. The kidneys were removed, weighed, and fixed in 10% formalin for periodic acid—methenamine silver (PAM) staining, and parts of the remaining tissues were embedded in optimal cutting temperature compound (Sakura Finetechnical, Tokyo, Japan) and frozen immediately in acetone cooled on dry ice.

2.2. Immunofluorescent Staining of MT-1/-2 in Rat Kidney. Immunofluorescent staining was performed as previously described [19]. Renal expression of MT-1/-2 was detected using mouse anti-MT-1/-2 antibody (Dako, Carpinteria, CA) followed by Alexa Fluor 594 goat anti-mouse IgG (Invitrogen, Carlsbad, CA). To determine whether MT-1/-2 was localized in podocytes or proximal tubular epithelial cells, the sections were counterstained with guinea pig antinephrin antibody (Fitzgerald, Concord, MA) or rabbit antiaquaporin 1 antibody (Millipore, Billerica, MA), followed by Alexa Fluor 488 goat anti-guinea pig IgG or anti-rabbit IgG (Invitrogen), respectively. Fluorescence images were obtained using a fluorescence microscope (BX51; Olympus, Tokyo, Japan).

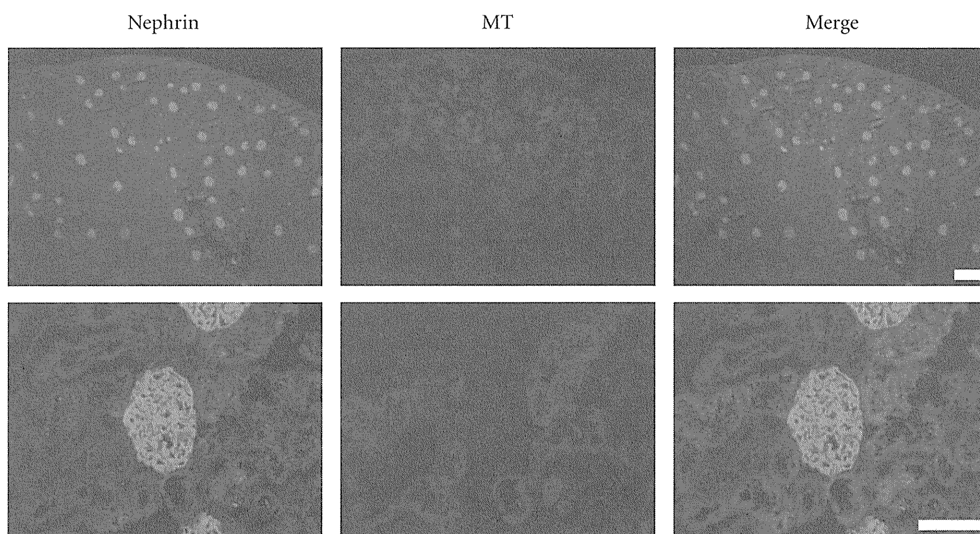
2.3. Cell Culture and Treatment. mProx24 cells, a murine renal proximal tubular epithelial cell line derived from



(a)

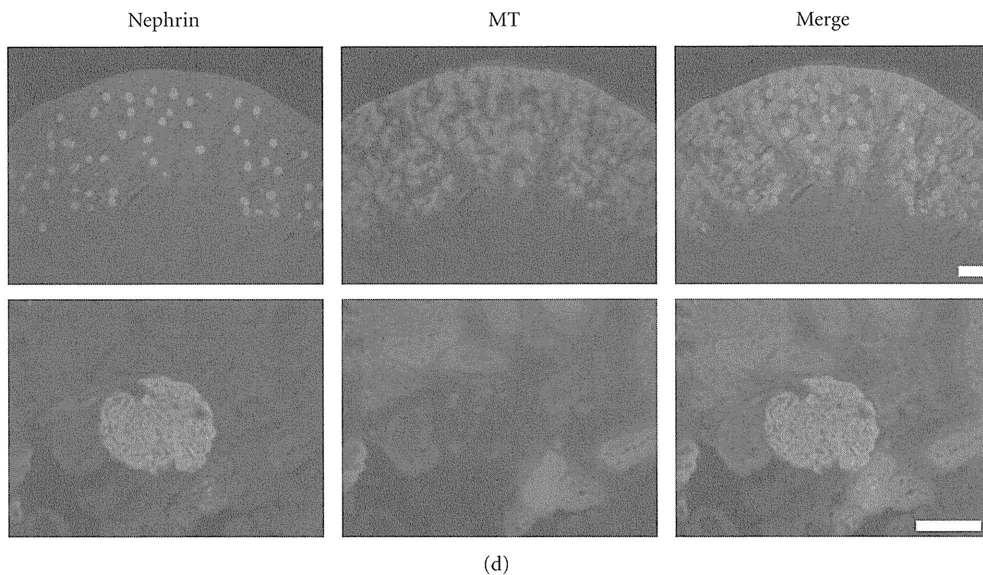


(b)



(c)

FIGURE 2: Continued.



(d)

Figure 2: MT-1/-2 expression in podocytes and proximal tubular cells of the kidney. Immunofluorescent staining was performed as described in *Materials and Methods*. Eight weeks after inducing diabetes, MT-1/-2 was predominantly expressed in the proximal tubular epithelial cells of the kidney (b) and weakly expressed in podocytes (d) in the kidney of diabetic rats. In control rats, MT-1/-2 was weakly expressed in proximal tubular epithelial cells (a), but hardly in the podocytes (c). AQP1: aquaporin 1, MT: MT-1/-2. Scale bar: upper panels, 200 μm; lower panels, 50 μm.

C57BL/6J adult mouse kidney [20], were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich Corp.) supplemented with 1000 mg/L D-glucose, 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in 5% CO_2. To evaluate the effect of high glucose on MT expression, the cells were serum-starved by culture in 0.5% FBS for 24 h, then stimulated with 4500 mg/L D-glucose (high glucose) or D-mannitol (Sigma-Aldrich Corp.) for 24 h. For antioxidant treatment, the cells were pretreated with vitamin E (Sigma-Aldrich Corp.) at concentration ranges from 20 to 200 nM for 24 h, then stimulated with high glucose for 24 h. Individual experiments were repeated at least three times with different lots or preparations of cells.

2.4. Quantitative Analyses of MT-1 Gene and MT-1/-2 Protein Expression in mProx Cells. RNA was isolated from mProx cells using an RNeasy Mini kit (Qiagen, Valencia, CA). Single-strand cDNA was synthesized from the extracted RNA using a RT-PCR kit (Perkin Elmer, Foster City, CA). To evaluate the mRNA expression of MT-1 in mProx24 cells, quantitative RT-PCR (qRT-PCR) was performed using StepOnePlus (Applied Biosystems, Tokyo, Japan) and Fast-Start SYBR Premix Ex Taq II (Takara Bio Inc., Otsu, Japan). The primers for the MT-1 gene (upstream 5′-TCTAAGCGTCACCACGACTTCA-3′ and downstream 5′-GTGCACTTGCAGTTCTTGCAG-3′) were purchased from Takara Bio Inc. Each sample was analyzed in triplicate and normalized for GAPDH mRNA expression. Immunofluorescent staining of MT-1/-2 protein was performed as described above. The immunofluorescence intensity in cultured mProx cells was calculated using the formula, x (density) $\times$ positive area (μm^2), using Lumina Vision software (Mitani Corporation).

2.5. Statistical Analysis. All values are means ± SEM. Statistically significant differences between groups were examined using one-way ANOVA followed by Scheffé's test. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. MT-1/-2 Expression Was Increased in Diabetic Kidney. MT-1/-2 expression was observed in the renal cortex from 1 week after the induction of diabetes. Its expression increased gradually and was strongly upregulated at week 8 (Figure 1,(d),(e),(f)). In contrast, MT-1/-2 was hardly detected in the kidney of control rats (Figure 1, (a),(b),(c)). Renal sections counterstained with antiaquaporin 1 and antinephrin antibodies revealed that MT-1/-2 expression was predominantly localized in the proximal tubular epithelial cells (Figure 2(b)), and to a lesser extent in the podocytes of the diabetic kidneys (Figure 2(d)). In control rats, MT-1/-2 was weakly expressed in the proximal tubular epithelial cells (Figure 2(a)), but not in the podocytes (Figure 2(c)). Body weight, kidney weight, UAE, and HbA1c are shown in Table 1. Diabetic rats had a significantly lower body weight and higher kidney weight per body weight at 8 weeks, but not at 1 and 2 weeks after the induction of diabetes. Similarly, The UAE and HbA1c level in the diabetic rats was significantly higher than in the control rats at 8 weeks, but not at 1 and 2 weeks. Glomerular hypertrophy and mesangial matrix expansion, but not interstitial changes and tubular atrophy

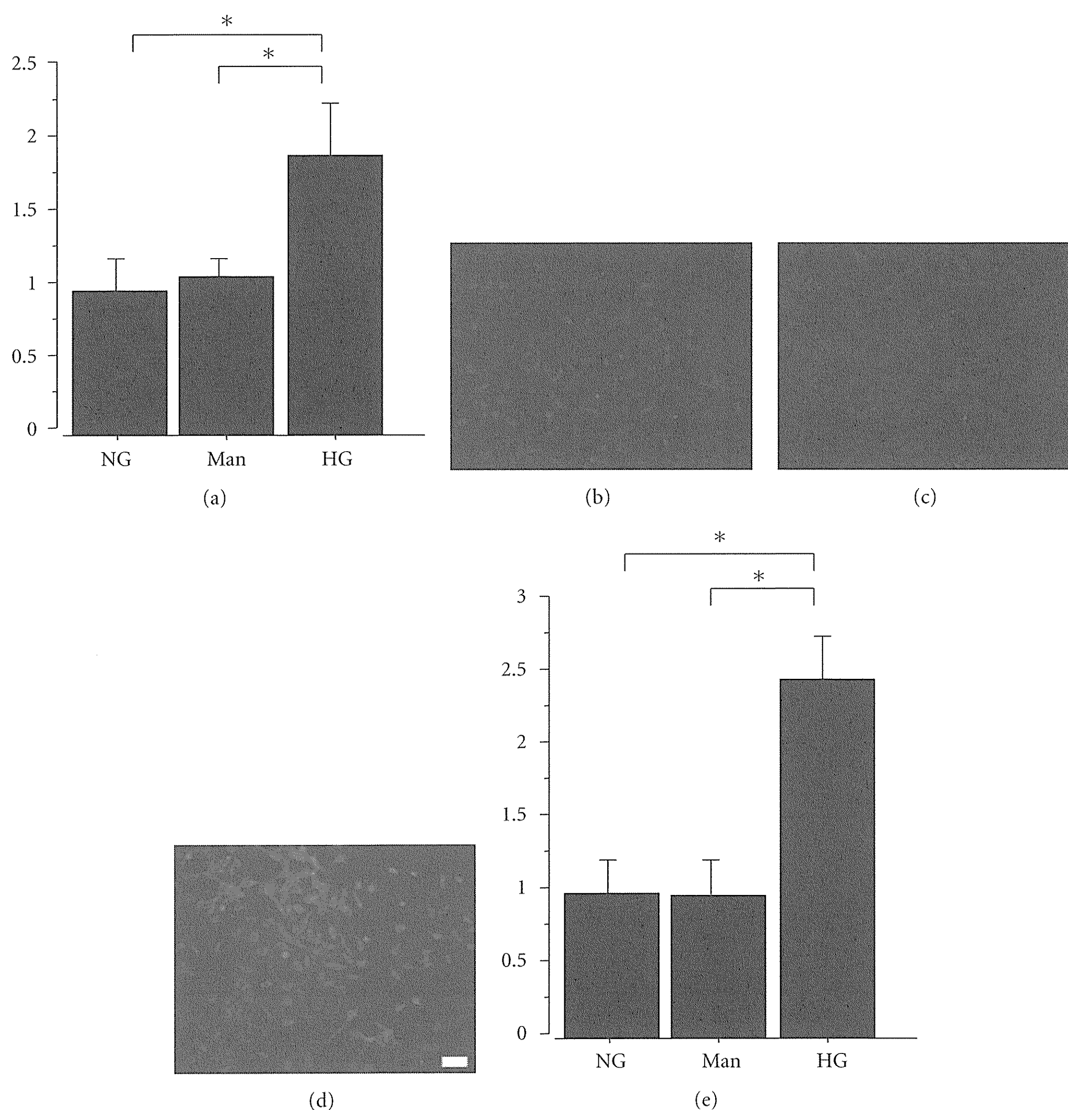


FIGURE 3: High glucose increases MT-1 mRNA and MT-1/-2 protein expression. mProx24 cells were serum-starved for 24 h before stimulation with high glucose or mannitol. (a) Cells were harvested after 24 h, and MT-1 mRNA expression was analyzed by qRT-PCR in three independent experiments and normalized for GAPDH. (b–e) MT-1/-2 protein expression was determined by immunofluorescent staining with anti-MT-1/-2 antibody 24 h after stimulation followed by densitometric analysis. Results are means ± SEM of three independent experiments. $^*P < 0.05$ versus high glucose; NG: normal glucose; Man: mannitol; HG: high glucose. Scale bar: 100 μm.

were observed in the diabetic rats as compared with control rats at 8 weeks (data not shown).

3.2. High Glucose Increased MT-1/-2 Expression in mProx24 Cells. qRT-PCR analyses revealed that exposure to the high glucose medium significantly increased MT-1 mRNA expression in mProx24 cells compared with normal glucose medium (Figure 3(a)). Similarly, high glucose, but not mannitol, significantly increased MT-1/-2 protein expression in mProx24 cells (Figures 3(b)–3(e)). These data indicate that high glucose increases the mRNA and protein expression of MT-1/-2 in mProx24 cells.

3.3. MT-1/-2 Expression Was Suppressed by Vitamin E. It is well known that high glucose increases the generation of ROS in various cells. To investigate the mechanism by which MT is induced by ROS in the high glucose condition, we examined the effects of an antioxidant, vitamin E, on MT-1/-2 expression in mProx24 cells. As shown in Figure 4, high-glucose-stimulated MT-1/-2 expression was significantly attenuated by vitamin E in a dose-dependent manner (Figure 4). Accordingly, these findings suggest that ROS generated by high glucose induces MT-1/-2 expression in the proximal tubular epithelial cells of the kidney.

4. Discussion

There is increasing evidence from experimental and clinical studies to suggest that oxidative stress plays a critical role in the pathogenesis and progression of diabetic

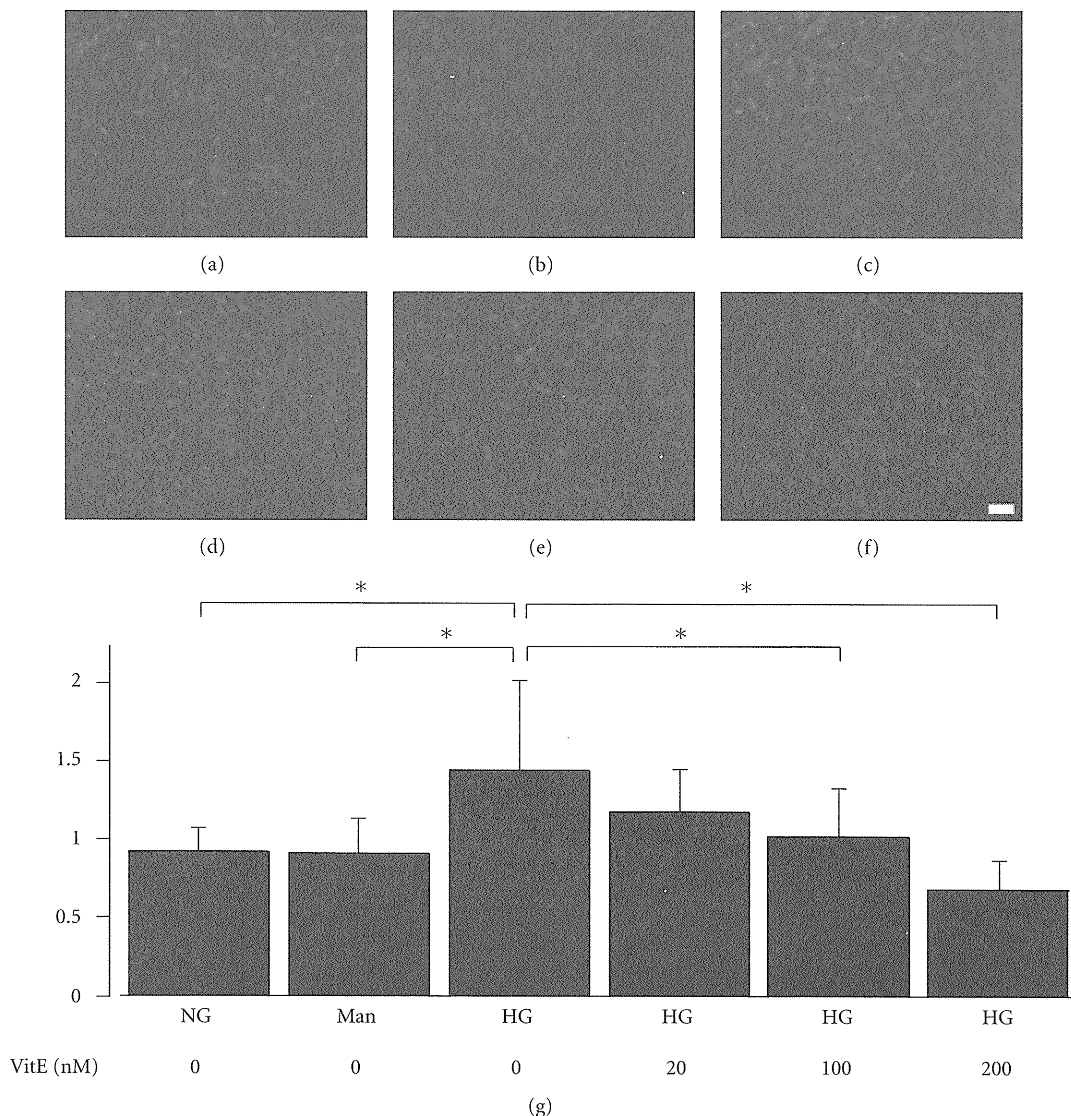


FIGURE 4: Vitamin E suppresses high glucose-induced MT-1/-2 expression. mProx24 cells were serum-starved and pretreated with vehicle or vitamin E for 24 h before stimulation with high glucose or mannitol. MT-1/-2 expression was determined by immunofluorescent staining. MT-1/-2 expression was not increased by mannitol (b) compared with normal glucose (a), but was increased by high glucose (c). High glucose-induced MT-1/-2 expression was attenuated by vitamin E pretreatment in a dose-dependent manner (d: 20 nM; E: 100 nM; F: 200 nM). The cells depicted are representative of three independent experiments. (g) Densitometric quantification of MT-1/-2 immunofluorescence. Results are means ± SEM of three independent experiments. $^*P < 0.05$ versus high glucose; NG: normal glucose; Man: mannitol; HG: high glucose; Vit E: vitamin E. Scale bar: 100 μm.

complications [21]. Since MT is a potent, endogenous and inducible antioxidant in various tissues [11, 12], we hypothesized that MT may be induced and act as an antioxidant in STZ-induced diabetic kidneys. Here, we found that high glucose induces the expression of MT-1/-2 mainly in proximal tubular epithelial cells and, to a lesser extent, in podocytes in rat kidneys. MT-1/-2 was dramatically expressed in renal proximal tubular epithelial cells within 1 week after inducing diabetes and gradually increased to week 8. MT-1/-2 expression seems to correlate with glucose level, but not with UAE, HbA1c, interstitial abnormalities. To our knowledge, this is the first report describing the localization and expression of MT-1/-2 in the diabetic kidney.

To elucidate the mechanism by which diabetes induces MT-1/-2 expression in proximal tubular epithelial cells, we investigated the effects of high glucose stimulation on mProx24, a murine renal proximal tubular epithelial cell line. We detected increased MT-1 mRNA and MT-1/-2 protein expression in the high glucose condition and found that high glucose-induced MT-1/-2 expression was suppressed by pretreatment with the antioxidant vitamin E. Vitamin E is well known to have high biological activity to protect cells from the propagation of free radical reactions [22, 23], thus we chose vitamin E in this study. These data suggest that ROS and oxidative stress, which are induced by high glucose, may be involved in the induction of MT-1/-2. Although

Table 1: Metabolic data at 1, 2, and 8 weeks after inducing diabetes.

	1 week	2 week	8 week
Body weight (g)			
Control	204 ± 6.3	241 ± 10.4	380 ± 13.3
Diabetic	198 ± 4.7	225 ± 11.5	248 ± 16.6*
Kidney weight (mg/g BW)			
Control	5.8 ± 0.4	5.6 ± 0.8	4.5 ± 0.7
Diabetic	5.9 ± 0.6	6.1 ± 1.0	6.7 ± 0.9*
UAE (μg/day)			
Control	110 ± 7.3	121 ± 8.1	137 ± 14.7
Diabetic	116 ± 5.7	125 ± 9.4	458 ± 24.5*
HbA1c (%)			
Control	3.7 ± 0.4	3.8 ± 0.6	3.8 ± 0.5
Diabetic	3.8 ± 0.3	4.3 ± 0.7	7.8 ± 0.9*

Data are means ± SEM; $^*P < 0.05$ versus the control group. BW: body weight; UAE: urinary albumin excretion; HbA1c: hemoglobin A1c.

several studies have shown that MT protein expression is increased in the kidney of diabetic animals [16–18], the cellular distribution of MTs has not been addressed. Our data provide the first evidence for the expression profile of MT-1/-2 in the diabetic kidney. We speculate that MT-1/-2 is highly induced in proximal tubular epithelial cells in compensation for oxidative stress induced by high glucose.

Our study has potential limitations. First, we speculated that MT-1/-2 expression was upregulated by ROS, but further studies are needed to elucidate the underlying mechanisms. Although Zn is known to induce the gene and protein expression of MTs [24], this essential trace element is unlikely to be involved in our findings because the same chow was provided to the control and diabetic rats. In this study, we showed that high-glucose-stimulated MT-1/-2 expression was attenuated by vitamin E *in vitro*, but we have no data about diabetic rats treated by vitamin E. MT-1/-2 expression in the diabetic state may differ between cells and tissues, and the mechanisms by which other antioxidants regulate the expression of MT remain unclear. Further studies are needed to elucidate these issues. Second, it is still controversial whether site-specific induction of MT plays an important role in diabetic nephropathy. Podocyte-specific overexpression of MT reduced diabetic nephropathy in transgenic mice [25]. However, no studies have investigated whether MT expression in proximal tubular epithelial cells has a protective effect in diabetic animal models. Therefore, diabetes models using MT-knockout mice are needed to answer this question.

In conclusion, renal ROS, which are induced by diabetes, upregulate MT-1/-2 expression in proximal tubular epithelial cells of the kidney. Our results suggest that MT-1/-2 might be a novel therapeutic target to treat diabetic nephropathy.

Acknowledgments

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References

[1] A. E. Declèves and K. Sharma, "New pharmacological treatments for improving renal outcomes in diabetes," *Nature Reviews Nephrology*, vol. 6, no. 6, pp. 371–380, 2010.

[2] J. W. Baynes and S. R. Thorpe, "Role of oxidative stress in diabetic complications: a new perspective on an old paradigm," *Diabetes*, vol. 48, no. 1, pp. 1–9, 1999.

[3] T. Nishikawa, D. Edelstein, X. L. Du et al., "Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage," *Nature*, vol. 404, no. 6779, pp. 787–790, 2000.

[4] T. Matsuoka, J. Wada, I. Hashimoto et al., "Gene delivery of Tim44 reduces mitochondrial superoxide production and ameliorates neointimal proliferation of injured carotid artery in diabetic rats," *Diabetes*, vol. 54, no. 10, pp. 2882–2890, 2005.

[5] P. A. Craven, M. F. Melhem, S. L. Phillips, and F. R. DeRubertis, "Overexpression of Cu^{2+}/Zn^{2+} superoxide dismutase protects against early diabetic glomerular injury in transgenic mice," *Diabetes*, vol. 50, no. 9, pp. 2114–2125, 2001.

[6] F. R. DeRubertis, P. A. Craven, M. F. Melhem, and E. M. Salah, "Attenuation of renal injury in db/db mice overexpressing superoxide dismutase: evidence for reduced superoxide-nitric oxide interaction," *Diabetes*, vol. 53, no. 3, pp. 762–768, 2004.

[7] D. H. Hamer, "Metallothionein," *Annual Review of Biochemistry*, vol. 55, pp. 913–951, 1986.

[8] M. Vasak, "Advances in metallothionein structure and functions," *Journal of Trace Elements in Medicine and Biology*, vol. 19, no. 1, pp. 13–17, 2005.

[9] P. J. Thornalley and M. Vasak, "Possible role for metallothionein in protection against radiation-induced oxidative stress. Kinetics and mechanism of its reaction with superoxide and hydroxyl radicals," *Biochimica et Biophysica Acta*, vol. 827, no. 1, pp. 36–44, 1985.

[10] R. D. Palmiter, "The elusive function of metallothioneins," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 15, pp. 8428–8430, 1998.

[11] Y. J. Kang, Y. Chen, A. Yu, M. Voss-McCowan, and P. N. Epstein, "Overexpression of metallothionein in the heart of transgenic mice suppresses doxorubicin cardiotoxicity," *Journal of Clinical Investigation*, vol. 100, no. 6, pp. 1501–1506, 1997.

[12] A. R. Quesada, R. W. Byrnes, S. O. Krezoski, and D. H. Petering, "Direct reaction of H_2O_2 with sulfhydryl groups in HL-60 cells: zinc- metallothionein and other sites," *Archives of Biochemistry and Biophysics*, vol. 334, no. 2, pp. 241–250, 1996.

[13] M. Ebadi, H. Brown-Borg, H. El Refaey et al., "Metallothionein-mediated neuroprotection in genetically engineered mouse models of Parkinson's disease," *Molecular Brain Research*, vol. 134, no. 1, pp. 67–75, 2005.

[14] I. Miyazaki, M. Asanuma, H. Hozumi, K. Miyoshi, and N. Sogawa, "Protective effects of metallothionein against dopamine quinone-induced dopaminergic neurotoxicity," *FEBS Letters*, vol. 581, no. 25, pp. 5003–5008, 2007.

[15] I. Miyazaki, M. Asanuma, Y. Kikkawa et al., "Astrocyte-derived metallothionein protects dopaminergic neurons from