glomerular and tubulointerstitial tissues, and exudative lesions [5]. Among these diabetic lesions, specific glomerular lesions in advanced human diabetic nephropathy include nodular lesions, arteriolar lesions (both efferent and afferent arterioles), and doughnut lesions. Nodular lesions are of importance for advanced diabetic nephropathy, which correlates with massive proteinuria in clinical settings. Nodular lesions were first described by Kimmelstiel and Wilson in 1936 [6]. In contrast, nodular lesions are hardly observed in experimental diabetic models showing high glucose levels without other insults. Insults in addition to glucose levels, such as endothelial cell nitric oxide synthase (eNOS) deficiency [7], integrin $\alpha 1$ deficiency [8] and the presence of Thy-1.1 nephritis [9], may be required for the formation of diabetic nodular lesions. Therefore, deeper insights into the pathogenesis of diabetic advanced lesions, including nodular lesions, are required. However, pathogenesis of these specific lesions in advanced diabetic nephropathy remains to be fully investigated. Further studies would be required to determine the pathogenesis of nodular lesions, as well as other lesions characteristic to advanced diabetic nephropathy, such as exudative lesions.

Here in this manuscript, we focus on pathogenesis and lessons from animal models of nodular lesions characteristic to advanced human glomerular lesions, both in type 1 and 2 diabetic patients.

Nodular lesions in advanced human diabetic nephropathy

Nodular lesions in human diabetic nephropathy have been reported in 10–50 % of biopsy specimens in both type 1 and type 2 diabetes [5]. Nodular lesions contain extracellular matrix components, including type IV collagen, which exists in normal glomerular structure. Electron microscopic studies revealed that nodular lesions consisted of accumulation of mesangial matrix with visible collagen fibrils, fibrils of unknown origin, small lipid particles, and cell debris [10–12]. Nodular lesions appear to develop from the central area of a stalk of a glomerular tuft, and encroach upon the surrounding capillary lumens [12]. In contrast, we previously reported that mesangiolysis might play a role in the formation of nodular lesions, with the deposition of type VI collagen in glomeruli (Fig. 1) [13, 14]. Nodular lesions possibly associated with mesangiolysis sometimes contain laminated structure. Surrounding the strongly Periodic Acid Methenamine silver (PAM)-positive materials, weakly PAM-positive materials with circumferential lamination as nodular lesions were observed (Fig. 1). Type IV collagen was mainly detected in the strongly PAM-positive area, whereas type VI collagen was detected in the weakly PAM-positive area. In this study, we examined 355 kidney specimens obtained from 327 patients with primary diabetes mellitus [13]. This report hypothesizes that: (1) the mesangiolysis is the initial lesion occurring in glomeruli in the process of diabetic nodule formation, and disturbed blood flow into glomeruli, caused by diabetic arteriolosclerosis, may be implicated in the development of the mesangiolysis; and (2) concentric compression of the lysed mesangial matrix by recanalized capillaries forms layered structures and ultimately completed diabetic nodules [13]. Hong et al. reported that diabetic patients with biopsy-proven nodular lesions had longer diabetic durations, more severe renal damage, and exhibited a poorer kidney prognosis. Furthermore, patients with nodular lesions more frequently revealed evidence of diabetic retinopathy [15]. Supporting this notion, Schwartz et al. [16] described that patients with Kimmelstiel–Wilson nodules had elevated serum creatinines and more severe overall retinopathy than those without Kimmelstiel–Wilson nodules. In contrast, kidney biopsy specimens from patients at presentation without overt clinical manifestations of glucose intolerance revealed typical nodular glomerulosclerosis, a negative deposition based on an immunofluorescence study, and neither any significant electron dense deposits nor fibrils on electron microscopy [17]. In this study, Sanai et al. concluded that diabetic nodular glomerulosclerosis occurred in patients without overt diabetes, suggesting the role of factors additional to prolonged hyperglycemia in the pathogenesis of this disorder. Interestingly, supporting this notion, nodular lesions have been also observed in membranoproliferative glomerulonephritis, amyloidosis, and light-chain deposition disease without diabetes [5]. In addition, nodular lesions are also observed in Goodpasture's syndrome [18]. Among these diseases, the strongly PAM-positive materials increased in glomeruli. However, mesangiolysis is not always observed in patients with nodular lesions with or without diabetic conditions. We previously reported nodular lesions in diabetes coexisting with mesaigiolysis or without mesangiolysis [13], although underlining mechanisms remain to be investigated. Further studies to determine mechanisms involved in the pathogenesis of nodular lesions with or without diabetes would be required.

Nodular-like lesions and mesangiolysis in an experimental model

To address the hypothesis that vascular endothelial injury and mesangiolysis are closely related to the formation of nodular lesions in diabetes, we attempted to induce nodular-like lesions resembling those seen in human patients by administration of monocrotaline [19]. In this study, marked mesangial expansion was observed in Otsuka Long-Evans

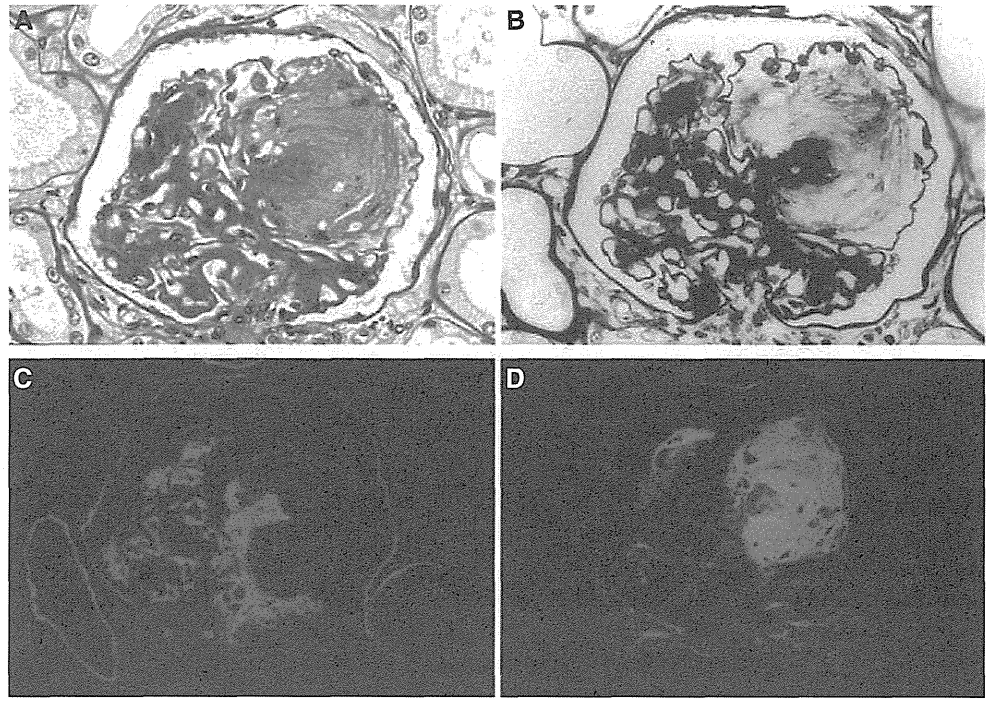

Fig. 1 Human diabetic nodular lesions by light microscopy, using serial sections. Surrounding the strongly Periodic Acid Methenamine silver (PAM)-positive materials, weakly PAM-positive materials with circumferential lamination as nodular lesions were observed (**a** Periodic Acid-Schiff (PAS) stain, ×250, **b** PAM stain, ×250). Type IV collagen was mainly detected in the strongly PAM-positive area (**c** ×250), and type VI collagen in the weakly PAM-positive area (**d** ×250). (From Ref. [14], reproduced with permission from Japanese Society of Nephrology)

Tokushima Fatty (OLETF) rats, in contrast to Long-Evans Tokushima Otsuka (LETO) rats, used as controls, after monocrotaline injection. Moreover, some glomeruli showed typical mesangiolytic lesions and nodular-like lesions in monocrotaline-treated OLETF rats at 50 weeks, concomitant with glomerular capillary endothelial cell swelling and nodular-like lesions and mesangiolysis gradually increased in monocrotaline-treated OLETF rats (Fig. 2) [19]. Matrix metalloproteinase (MMP)-2 and membrane-type 1 (MT1)-MMP proteins increased in the expanded mesangial lesions in OLETF rats as compared to LET rats. Gelatin zymography revealed an increase in 62-kDa activated MMP-2 in the culture supernatants of isolated glomeruli from OLETF rats. In situ enzymatic activity of MMP in the mesangial areas was also detected in 50-week-old monocrotaline-injected OLETF rats. The most compelling part of our study was proceeding to mesangiolysis under hyperglycemic conditions, possibly followed by the formation of nodular-like lesions. These results suggest that diabetic conditions and some toxic events associated with monocrotaline injection during the process of reconstruction from mesangiolysis may be required for the formation of nodular-like lesions resembling human ones in this model, although the lamination of nodules in this model was incomplete.

Nodular-like lesions in experimental models

The Animal Models of Diabetic Complications Consortium (AMDCC), created in 2001, describes research criteria for validating a progressive mouse model of diabetic nephropathy [20]. Especially, pathology of kidneys defines criteria consisting of four issues: (1) advanced mesangial matrix expansion ± nodular sclerosis and mesangiolysis; (2) any degree of arteriolar hyalinosis; (3) glomerular basement membrane thickening by > 50 % over baseline; and (4) tubulointerstitial fibrosis. AMDCC investigators conclude that no current model meets them all. Therefore, the criteria should be viewed as goals, rather than requirements [20].

A few reports have described experimental diabetic models resembling human nodular lesions. Inagi et al. [21] utilized megsin overexpression in a receptor for advanced glycation end products (RAGE) and inducible nitric oxide synthase (iNOS) transgenic mice. In this particular model, the triple transgenic mice overexpressing megsin, RAGE, and iNOS developed, at an early age (16 weeks), severe albuminuria and kidney damage, characterized by development of mesangial expansion, and tubulointerstitial damage, with all of the characteristics of human diabetic nephropathy. In addition, 30–40 %

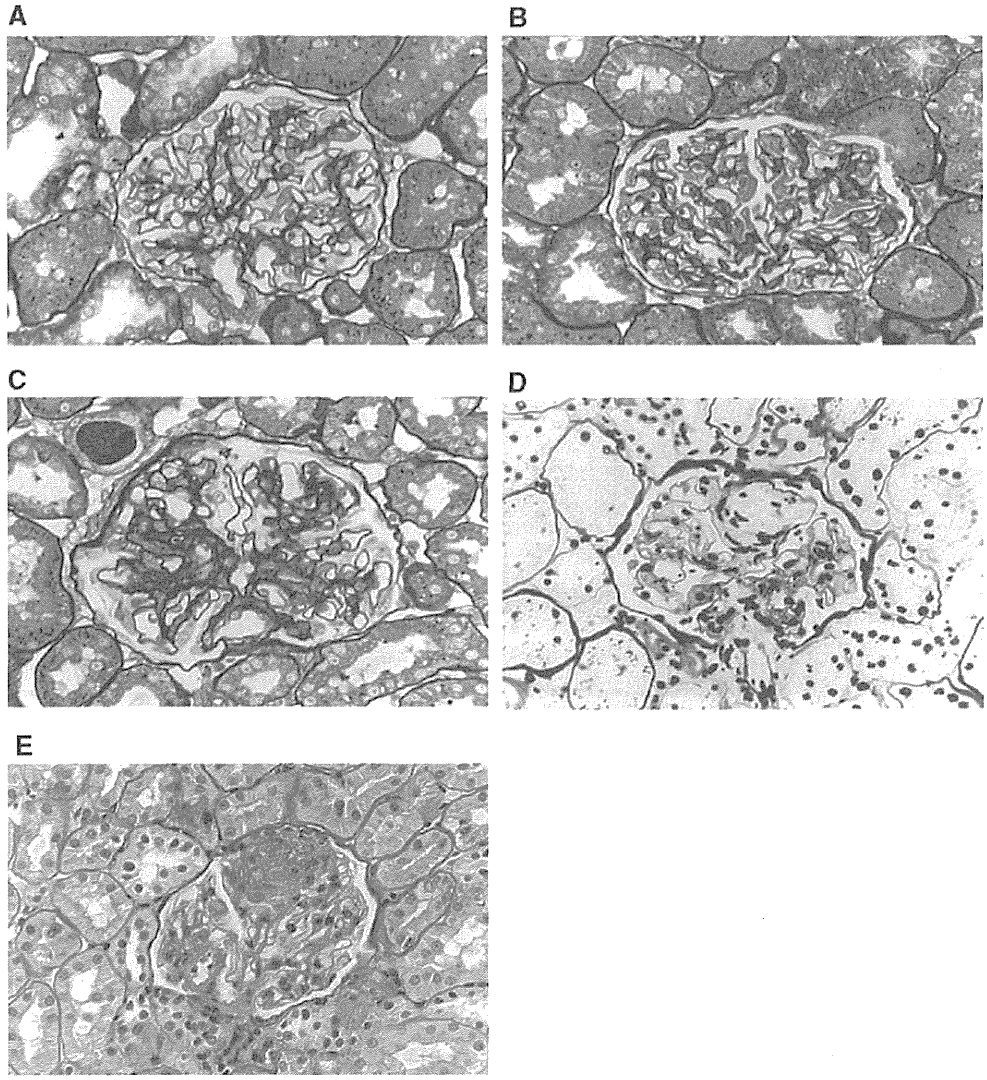

Fig. 2 Mesangiolysis and diffuse glomerulosclerosis detected in monocrotaline-injected Otsuka Long-Evans Tokushima Fatty (OLETF) rats. Representative kidney tissues obtained from a saline-injected Long-Evans Tokushima Otsuka (LETO) rat (50 weeks **a**) and a saline-injected OLETF (50 weeks **c**). A monocrotaline-injected LETO (50 weeks **b**), and a monocrotaline-injected OLETF (50 weeks **d**, **e**) showing mesangiolysis and nodular-like lesions. **a**–**d** PAM stain, ×400; **e** PAS stain, ×400. (From Ref. [19], reproduced with permission from The European Renal Association, European Dialysis and Transplant Association)

of glomeruli exhibit nodule-like lesions with an increase in local oxidative stress, which may be relevant to previous reports describing the possible involvement of oxidative stress [22]. Further, endothelial nitric oxide synthase (eNOS) (−/−) mice, backcrossed to C57BLKS/J db/db mice, exhibited dramatic albuminuria, arteriolar hyalinosis, increased glomerular basement membrane thickness, mesangial expansion, mesangiolysis, and focal segmental and early nodular glomerulosclerosis by 26 weeks, although the severity of hyperglycemia was similar to C57BLKS/J db/db mice [7]. Remarkably, eNOS(−/−) C57BLKS db/db exhibited decreases in GFR to levels < 50 % of that in eNOS(+/+) C57BLKS db/db. Similarly, The $eNOS^{-/-}/lepr^{db/db}$ double-knockout mice showed significant glomerular injury, including mesangiolysis, microaneurysms, increased mesangial matrix expansion with nodular lesions (nodular glomerulosclerosis) and globally sclerotic glomeruli [23]. Further, in streptozotocin-induced eNOS knockout mice, deficiency of eNOS-derived NO causes glomerular endothelial injury in the setting of diabetes and results in overt albuminuria and glomerular mesangiolysis and nodular-like lesions in nephropathy-resistant inbred C57BL6 mice [24]. Supportingly, the level of eNO may be one of the critical determinants for whether patients with diabetes are at risk for developing nephropathy, including nodular-like lesions [25].

Molecular mechanisms possibly involved in nodular lesions

Putative promoters of progression of diabetic nephropathy include systemic blood pressure, glomerular hypertension, proteinuria, glycemic control, renin–angiotensin–aldosterone system, hyperlipidemia, obesity, dietary protein intake, smoking, oligonephropathy, and angiotensin-converting enzyme ID polymorphism [1]. In addition to these promoters, recent studies reveal that inflammatory processes may play a key role in the progression of advanced diabetic nephropathy [26, 27]. Pro-inflammatory cytokines and chemokines have been reported to contribute to the progression of advanced diabetic kidney lesions. Supporting this notion, urinary levels of monocyte chemoattractant protein (MCP)-1/CCL2, a prototype of chemokine, increased in accordance with the damage of human glomerular diffuse lesions through infiltration and activation of macrophages [27]. In addition, there was a significant correlation between urinary MCP-1 levels and the presence of nodular lesion and mesangiolysis. Moreover, the mitogen-activated protein kinase (MAPK) phosphorylation, deeply involved in production and signal transduction of pro-inflammatory cytokines and chemokines, contributes to human diabetic nephropathy, including the presence of nodular lesions [28]. In particular, extracellular signal-regulated kinase (ERK) may be distinctly involved in glomerular lesions in human diabetic nephropathy. Interestingly, Thy-1.1 nephritis aggravated diabetic nephropathy, leading to the formation of nodular-like lesions [9]. Mesangiolysis and macrophage activation, accompanied by mesangial damage, are essential in this particular model. Collectively, the interaction between kidney resident cells and infiltrating cells enhances the synthesis matrix protein, cytokines/chemokines and pro-fibrotic growth factors, which may promote and escalate chronic inflammatory processes, thereby perpetuating kidney fibrosis, including advanced diabetic nephropathy [29–32].

We previously reported that diabetic glomerular lesions were formed during the reconstruction process of mesangiolysis. In this setting, matrix metalloproteinase (MMP)-2, which is activated from proMMP-2 by membrane-type (MT)-MMP, was produced and activated in glomeruli through the progression of diabetic nephropathy in a type 2 diabetic model, OLETF rats [19]. These findings suggest matrix turnover regulated by MMPs, and their modulators may have some effect on the remodeling of the glomerular matrix in diabetic nephropathy.

In close relation to inflammatory processes, advanced glycation end-products (AGEs) seem to play a central role in the progression of diabetic nephropathy [33]. AGEs are localized most notably in nodular lesions, suggesting that advanced glycations may play a role in the progression of diabetic nephropathy through impairment of the assembly of matrix proteins in vivo. Because type V and type VI collagens are the major components of nodular lesions, increases in these interstitial and fibril or microfibril collagens may contribute to the formation of wider strands in the mesangial matrix of a nodular lesion. As no metalloprotease that is specific for type VI collagen has been identified thus far, AGE formation might occur preferentially in type VI collagen-rich nodular lesions, which are sites of slow turnover [33]. These results may be relevant to our previous observation [13, 19, 22] (Fig. 3).

Further, endothelial dysfunction that accompanies a diabetic state may cause advanced diabetic nephropathy, including nodular-like lesions [7, 23–25]. Endothelial damage induced by eNOS deficiency may be responsible for an uncoupling of the VEGF-eNO axis, resulting in increased levels of VEGF and excessive endothelial cell proliferation, thereby coupled with altered autoregulation consequent to the development of preglomerular arteriolar disease [25]. Therefore, together with our previous studies in humans [13] and an experimental model [19], insults resulting in endothelial damage may be one of the important determinants for whether patients with diabetes are at risk for developing advanced nephropathy.

More recently, novel mechanisms involved in anti-inflammatory effects in the progression of diabetic nephropathy have been reported. Podocyte-specific overexpression of human angiotensin-converting enzyme 2 attenuated diabetic nephropathy in mice [34]. Further, hepatocyte growth factor gene therapy enhanced renal expression of stromal-cell-derived factor-1 and was subsequently associated with an increased number of bone-marrow-derived cells getting into the injured kidneys in db/db mice [35]. Interestingly, these cells were mainly monocyte-derived macrophage, which may contribute to kidney tissue repair and regeneration. In contrast, cholecystokinin suppressed the activation of macrophage and expression of pro-inflammatory genes in diabetic kidneys [36].

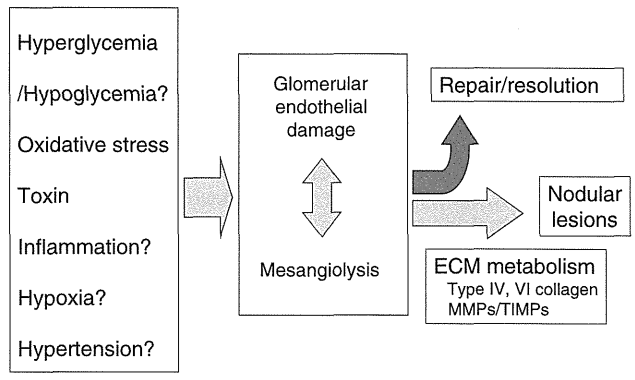

Fig. 3 Possible mechanism in nodular lesions associated with mesangiolysis

Collectively, further studies would be required to determine the precise mechanisms involved in formation and progression of nodular lesions and mesangiolysis, including origin and roles of macrophages, key players in the progression of advanced diabetic lesions.

Concluding remarks and future directions

We have introduced the pathological features and experimental models of diabetic nodular lesions. Because diabetic nodular lesions are of importance in the progression of advanced human diabetic nephropathy, it is essential that the pathogenesis of nodular lesions and mesangiolysis be elucidated in near future. This may provide a key not only for better understanding, but for the therapeutic benefit of diabetic nephropathy.

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Association between urinary angiotensinogen levels and renal and cardiovascular prognoses in patients with type 2 diabetes mellitus

Makoto Sawaguchi[1], **Shin-ichi Araki**[1,*], **Hiroyuki Kobori**[2], **Maki Urushihara**[2], **Masakazu Haneda**[3], **Daisuke Koya**[4], **Atsunori Kashiwagi**[1], **Takashi Uzu**[1], and **Hiroshi Maegawa**[1]

[1]Department of Medicine, Shiga University of Medical Science, Otsu, Shiga

[2]Departments of Medicine and Physiology, Tulane University Health Sciences Center, New Orleans, LA, USA

[3]Department of Medicine, Asahikawa Medical College, Asahikawa, Hokkaido

[4]Department of Medicine, Kanazawa Medical University, Kahoku-gun, Ishikawa, Japan

Abstract

Aims/Introduction—Activation of the renin-angiotensin system (RAS) in the kidney plays an important role in renal function. The aim of this study was to investigate whether plasma and urinary angiotensinogen levels were associated with renal and cardiovascular prognosis in type 2 diabetic patients.

Materials and Methods—We measured plasma and urinary angiotensinogen levels in the observational follow-up cohort of 234 Japanese type 2 diabetic patients (144 with normoalbuminuria, 90 with albuminuria) enrolled between 1998 and 1999 and followed them up until the end of 2008. The associations of these markers with the annual decline in the estimated glomerular filtration rate (eGFR) and incidence of renal and cardiovascular composite endpoints (chronic hemodialysis, myocardial infarction, angina pectoris, stroke and cerebral hemorrhage) were evaluated.

Results—At baseline, urinary angiotensinogen levels correlated with urinary albumin-creatinine ratio, urinary β_2-microglobulin and inversely with eGFR. In contrast, plasma angiotensinogen levels correlated neither with these renal factors nor with urinary angiotensinogen levels. In the follow-up study (median duration: 9 years), urinary angiotensinogen, but not plasma angiotensinogen, correlated inversely with the annual change in eGFR ($r = -0.51$, $P < 0.001$).When patients were divided into four subgroups according to albuminuria and urinary angiotensinogen levels, patients with albuminuria and high urinary angiotensinogen levels showed a progressive decline of eGFR and a higher incidence of renal and cardiovascular composite endpoints.

Conclusions—These results suggest that the higher level of urinary angiotensinogen in type 2 diabetic patients with albuminuria is a high risk factor for worsening renal and cardiovascular complications.

*Corresponding author. Shin-ichi Araki Tel: +81-77-548-2222 Fax: +81-77-543-3858, araki@belle.shiga-med.ac.jp.

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All authors have no conflict of interest to disclose.

Keywords

Angiotensinogen; Diabetes mellitus; Glomerular filtration rate

INTRODUCTION

Diabetic nephropathy is a representative disorder of chronic kidney disease (CKD) and a leading cause of end-stage kidney disease (ESKD). This disorder is also associated with high morbidity and mortality of cardiovascular disease (CVD)[1–3]. Thus, prevention of development and progression of this disorder is of clinical importance to improve prognosis in diabetic patients.

Numerous clinical trials have documented that inhibition of the renin-angiotensin system (RAS) in diabetic patients can slow the progressive decrease in glomerular filtration rate (GFR) and reduce cardiovascular mortality and morbidity[4–6]. Based on clinical evidence, inhibition of the RAS is currently the first line treatment for diabetic nephropathy[7]. These results also support the concept that activation of RAS in diabetic patients is an important pathogenic mechanism of renal and cardiovascular complications[7]. However, despite the beneficial effects of RAS inhibition, all patients do not always show an improvement in the prognosis of these complications. Therefore, it is important to identify patients at higher risk of poor prognosis and a proper estimation of the status of intrarenal RAS activation may provide crucial information.

The kidney contains all components of the RAS pathway including the production of angiotensinogen[8]. Thus, the kidney can locally produce angiotensin II (AngII) by a mechanism independent of circulating AngII, known as the classical RAS pathway[8]. Intrarenally-produced AngII is reported to play an important role in renal hemodynamics and function as a paracrine factor[9].

We recently developed a direct method to quantify human plasma and urinary angiotensinogen levels using enzyme-linked immunosorbent assays (ELISA)[10]. Using this new method, we recently reported that urinary angiotensinogen may be a potential biomarker of the severity of CKD and intrarenal RAS status in hypertensive patients in the cross-sectional studies[11,12]. However, it is still unclear whether urinary and plasma angiotensinogen levels can be used to predict deterioration of renal function and the incidence of cardiovascular disease in a long longitudinal cohort. In the present study, we measured plasma and urinary angiotensinogen levels using our new ELISA method, in Japanese patients with type 2 diabetes who were enrolled in our observational follow-up study[2]. We then investigated whether these markers associate with renal and cardiovascular prognosis.

MATERIALS AND METHODS

Study Population and Samples

Japanese patients with type 2 diabetes mellitus were recruited from among participants who were registered in the Shiga Prospective Observational Follow-up Study between 1998 and 1999[2]. After obtaining written informed consent, each individual provided a spot urine sample and a fasting blood sample at baseline. The plasma and urine samples were kept at −80°C if not analyzed immediately. Based on the level of urinary albumin– creatinine ratio (UACR) at baseline, patients were classified as having normoalbuminuria (UACR < 30 mg/g Cr), micro-albuminuria (30 ≤ UACR < 300 mg/g Cr), or overt proteinuria (UACR ≥ 300 mg/g Cr). Finally, 234 patients with normoalbuminuria (n = 144), microalbuminuria (n = 53)

and overt proteinuria ($n = 37$) were enrolled and were followed up until the end of 2008 or the incidence of the renal and cardiovascular composite endpoints. In this study, patients with microalbuminuria and overt proteinuria were combined together into those with albuminuria (diabetic nephropathy). The participants underwent standardized clinical examination and biochemical tests annually, during the follow-up period. In this study, the values of HbA1c were presented in National Glycohemoglobin Standardization Program values according to the recommendations of the Japanese Diabetes Society[13]. The study protocol and informed consent procedure were approved by the Ethics Committee of Shiga University of Medical Science.

Measurement of Plasma and Urinary Angiotensinogen Levels

The concentrations of angiotensinogen in plasma and urine samples at baseline were measured with human angiotensinogen ELISA, as reported previously[10]. The sensitivity of this assay is >0.31 ng/mL. The intra- and inter-assay coefficients of variation were 4.4 and 4.3%, respectively. The urinary concentrations of creatinine were measured simultaneously by the enzymatic method. The urinary level of angiotensinogen was expressed in μg/g Cr.

Follow-up Evaluation

To evaluate deterioration of renal function, we assessed the annual decline in estimated GFR (eGFR). eGFR was calculated using the simplified prediction equation proposed by the Japanese Society of Nephrology[14]: eGFR (mL/min/1.73m^2) = 194 × [age (years)]$^{-0.287}$ × [serum creatinine (mg/dL)]$^{-1.094}$ × 0.739 (for female). The serum concentration of creatinine was measured using the enzymatic method. The annual decline in eGFR over the course of the study was determined from the slope of the plot of all measurements of eGFR for each individual calculated by linear regression analysis and was expressed in mL/min/1.73 m^2/year.

We also investigated the incidence of the renal and cardiovascular composite endpoints, including myocardial infarction, angina pectoris, stroke and cerebral hemorrhage and initiation of chronic hemodialysis. Myocardial infarction was defined as a clinical presentation characterized by typical symptoms, electrocardiographic changes associated with an elevation of cardiac biomarkers and angiographic evidence of coronary thrombosis. Angina pectoris was defined as a history of typical chest pain and electrocardiographic changes compatible with ischemic heart disease or the detection of myocardial perfusion defects with exercise stress tests. Stroke and cerebral hemorrhage were defined as a persistent focal neurological symptom in which onset was sudden and was not due to trauma or a tumor and where the responsible lesion was detected by imaging studies.

Statistical Analysis

Data are expressed as mean ± SD or median (interquartile range). As compared between two groups, unpaired Student's *t*-test for continuous variables and chi-square test for categorical variables were applied. A comparison among three or more groups was performed by ANOVA with the Tukey-Kramer HSD test. Due to the skewed distribution, urinary angiotensinogen, UACR and urinary β_2-microglobrin (U-β_2MG) values were log-transformed before analysis. Pearson regression analysis was applied for analysis of the correlation between two variables, using logarithmic transformed values of non-normally distributed variables. A multivariate liner regression model was applied to evaluate the independency of factors that showed significant correlation in the univariate model. The cumulative incidences of renal and cardiovascular composite endpoints were estimated using Kaplan–Meier procedure and were compared by the log-rank test. The follow-up time was censored if any composite endpoint was observed or if the patient was unavailable for follow-up. Risk for renal and cardiovascular composite endpoint was evaluated by a Cox hazard regression model. A

forward stepwise procedure was used to select explanatory variables with statistically significant effects on the time to the incidence of the endpoint. All analyses were performed by the SPSS software package (version 11; SPSS Inc., Chicago, IL, USA) and JMP for Windows (version 8.0.2; SAS Institute Inc, Cary, NC, USA). A two-sided P value <0.05 was considered statistically significant.

RESULTS

Baseline Clinical Characteristics

Table 1 lists the clinical characteristics of patients at baseline stratified by the stage of nephropathy. Gender, duration of diabetes, body mass index (BMI), HbA1c, systolic blood pressure (SBP), diastolic blood pressure (DBP), use of RAS inhibitors, triglyceride, UACR, eGFR and U-β_2MG were different between the normoalbuminuria and albuminuria groups.

Correlation Between Plasma Angiotensinogen Level and Various Parameters at Baseline

Plasma angiotensinogen levels were not different between two groups (normoalbuminuria: 24.7 ± 5.3, albuminuria: 24.1 ± 5.4 μg/mL). Univariate regression analysis showed weak correlations between plasma angiotensinogen levels and BMI, waist-hip ratio, SBP, total cholesterol, HDL-cholesterol and triglyceride, and no correlation with UACR, eGFR and U-β_2MG (Table 2). Plasma angiotensinogen levels were not different between patients treated with RAS inhibitors and those without them (24.4 ± 5.3 vs 24.6 ± 5.6 μg/mL, $P = 0.97$). Interestingly, plasma angiotensinogen levels were significantly higher in females than males (26.7 ± 5.3 vs 22.6 ± 4.6 μg/mL, $P < 0.001$). However, plasma angiotensinogen levels did not correlate with UACR, eGFR and U-β_2MG even when patients were analyzed separately according to gender.

Correlation Between Urinary Angiotensinogen Level and Various Parameters at Baseline

In contrast to plasma angiotensinogen, urinary angiotensinogen levels were higher in patients with albuminuria (62.0 μg/g Cr [interquartile range: 25.4–146.5]) than in those with normoalbuminuria (17.5 μg/g Cr [11.4–28.2], $P < 0.001$). Univariate regression analysis showed that urinary angiotensinogen levels correlated positively with UACR and U-β_2MG and inversely with eGFR (Table 2). Interestingly, there was no correlation between urinary angiotensinogen and plasma angiotensinogen ($r = 0.08$, $P = 0.21$). Urinary angiotensinogen levels were higher in patients treated with RAS inhibitors (38 μg/g Cr [19–133]) than those without (22 μg/g Cr [13–42], $P = 0.001$). However, this difference was probably due to the different prescription rate of RAS inhibitors in the two groups (normoalbuminuria: 16%, albuminuria: 32%). When urinary angiotensinogen levels were compared according to the stage of nephropathy, those in each stage were not different between patients treated with RAS inhibitors and those without. Unlike plasma angiotensinogen, the urinary angiotensinogen level in males was similar to that in females. Multiple regression analysis identified UACR and U-β_2MG as the independent and significant factors that correlated with urinary angiotensinogen levels.

Correlation Between Angiotensinogen Level and Annual Decline in eGFR

To explore the predictive role of plasma and urinary angiotensinogen levels for renal dysfunction, we investigated the correlation between each angiotensinogen and the annual change in eGFR during the follow-up period (median: 9 years, interquartile range: 6–10 years). As shown in Figure 1, urinary angiotensinogen, but not plasma angiotensinogen ($r = 0.00$, $P = 0.99$), correlated inversely with the annual change in eGFR ($r = -0.51$, $P < 0.001$). As other factors, the annual decline in eGFR correlated strongly with UACR ($r = -0.65$, $P < 0.001$) and correlated weakly with triglyceride ($r = -0.28$, $P < 0.001$), HDL-cholesterol ($r =$

0.15, $P = 0.027$), HbA1c ($r = -0.22$, $P = 0.001$), eGFR at baseline ($r = 0.32$, $P < 0.001$), BMI ($r = -0.24$, $P < 0.001$), SBP ($r = -0.24$, $P < 0.001$) and DBP ($r = -0.23$, $P < 0.001$).

Urinary Angiotensinogen and Renal Dysfunction in Patients with Albuminuria

Albuminuria is well known to be a risk factor for renal dysfunction and cardiovascular disease in patients with type 2 diabetes. Based on the strong correlation between urinary angiotensinogen and UACR, it was difficult to determine the specific role of each parameter in renal dysfunction. Therefore, to explore the clinical utility of measuring urinary angiotensiongen, we investigated the predictive effect of the combination of urinary angiotensinogen and albuminuria on deterioration of renal function. For this purpose, patients were divided into four groups according to the median value of urinary angiotensinogen levels (median cut-off values: 24.7 μg/g Cr) and the presence of albuminuria (>30 mg/g Cr). The eGFR at baseline (mL/min/1.73 m^2) was 80 ± 15 in those with low levels of urinary angiotensinogen and normoalbuminuria (L + N, $n = 97$), 84 ± 14 in those with high levels of urinary angiotensinogen and normoalbuminuria (H + N, $n = 47$), 82 ± 19 in those with low levels of urinary angiotensinogen and albuminuria (L + A, $n = 21$) and 66 ± 27 in patients with high levels of urinary angiotensinogen and albuminuria (H + A, $n = 69$). Among the four subgroups, the annual decline in eGFR during the follow-up was significantly greater in the H + A subgroup than other subgroups ($P < 0.05$ vs all other subgroup, Figure 2).

Urinary Angiotensinogen and Renal-Cardiovascular Outcomes in Patients with Albuminuria

Finally, we evaluated the association between urinary angiotensinogen at baseline and the incidence of renal and cardiovascular composite endpoints. A total of 58 patients experienced any of the composite endpoints (17 for chronic hemodialysis, 10 for myocardial infarction, 18 for angina pectoris, eight for stroke and five for cerebral hemorrhage). The incidence rate of this endpoint was higher in patients with high levels of urinary angiotensinogen than those with low levels of urinary angiotensinogen (36% vs 14%, $\chi^2 = 15.5$, $P < 0.001$). Similarly, the incidence rate of this endpoint was higher in patients with albuminuria than those with normoalbuminuria (47% vs 11%, $\chi^2 = 37.6$, $P < 0.001$). As shown in Figure 3, the cumulative incidence among the four subgroups was the highest in the H + A subgroup (log rank test: $P < 0.001$ for trend). Multivariate Cox proportional hazard regression model with the forward stepwise procedure identified four predictors of renal and cardiovascular outcomes: the combination of urinary angiotensinogen and albuminuria (adjusted odds ratio 4.5 [95% CI: 2.1–9.5] in H + A subgroup, 3.4 [1.2–9.3] in L + A subgroup and 1.6 [0.6–4.4] in H + N subgroup, 1.0 [reference] in L + N subgroup), age (1.04 [1.00–1.08]), eGFR at baseline (0.97 [0.96–0.98]) and past history of CVD (1.90 [1.06–3.41]).

DISCUSSION

In this study, analysis of baseline data showed that urinary angiotensinogen levels correlated with UACR and U-β_2MG and inversely with eGFR. In contrast, plasma angiotensinogen levels did not correlate with these factors or with urinary angiotensinogen levels. Furthermore, follow-up analysis indicated that patients with albuminuria and high levels of urinary angiotensinogen showed the progressive decline of renal function and the high incidence of renal-cardiovascular endpoints. These results suggest that the higher level of urinary angiotensinogen in type 2 diabetic patients with nephropathy is a high risk factor for worsening renal and cardiovascular complications.

In the present study, urinary angiotensinogen levels correlated closely with renal factors but did not correlate with plasma angiotensinogen levels. In contrast, plasma angiotensinogen levels correlated with various metabolic factors including BMI, waist-hip ratio and serum lipids, in agreement with the data of a previous report[15], but they did not correlate with renal factors. These results suggest that urinary and plasma angiotensinogen are produced by different sources and play different roles in renal function. Although angiotensinogen is produced and secreted by the liver, it is also produced in the kidney[9]. Previous studies have investigated whether circulating angiotensinogen is a source of urinary angiotensinogen. In hypertensive and normotensive rats infused human angiotensinogen, the circulating human angiotensinogen was not detectable in the urine, indicating limited glomerular permeability and/or tubular degradation of circulating angiotensinogen[16]. In the kidney under normal conditions, the expression of angiotensinogen is reported to localize in proximal tubular cells and angiotensinogen produced in proximal tubular cells is considered to be directly released into the renal tubular lumen[9]. Under diabetic conditions, the expression of angiotensinogen is reported to be enhanced in proximal tubular cells and to be also observed in mesangial cells[17,18]. Some human studies reported higher levels of urinary angiotensinogen in diabetic patients than in control subjects and patients with non-diabetic kidney diseases[11,19], whereas plasma angiotensinogen levels were similar in diabetic patients and control subjects[19]. Because the kidney contains all components of the RAS pathway, the enhanced expression of intrarenal angiotensinogen may lead to the intrarenal RAS activation. Thus, these results suggest that urinary angiotensinogen is produced locally in the kidney, but not from plasma, and its levels may associate with intrarenal RAS activation in diabetic patients.

In the present study, patients with high levels of urinary angiotensinogen, not plasma angiotensinogen, showed a greater decline in eGFR during the follow-up. A similar observation in patients with CKD documented the presence of higher urinary angiotensinogen levels in patients with low eGFR and patients with higher levels of urinary angiotensinogen showed increased risk of renal dysfunction during a mean follow-up period of 23 months[20]. Thus, urinary angiotensinogen is considered to be associated with the deterioration of renal function in patients with CKD including diabetic nephropathy.

Albuminuria is well known to be not only a predictor of progression to ESKD but also a risk factor for cardiovascular disease[1,2]. In this study, urinary angiotensinogen levels correlated closely with UACR as well as previous reports[12,21]. However, patients with albuminuria and higher levels of urinary angiotensinogen showed a progressive decline in eGFR and the high incidence of renal-cardiovascular endpoints than those with albuminuria and low levels of angiotensinogen. Thus, the increase of urinary angiotensinogen in patients with albuminuria may predict the patients at risk for worsening renal and cardiovascular complications.

What is the mechanism by which urinary angiotensinogen levels associate with worsening renal and cardiovascular complications? In this study, urinary angiotensinogen levels correlated with UACR and U-β_2MG. Transgenic mice overexpressing angiotensinogen in renal proximal tubular cells were reported to develop albuminuria, hypertension and renal injury[22]. The induction of diabetes with streptozotocin in these transgenic mice enhanced the aforementioned abnormal changes and induced apoptosis of renal proximal tubular cells[23]. Although diabetic nephropathy was traditionally considered to cause glomerular damage primarily, it is now widely accepted that deterioration of renal function in diabetic patients correlates with the degree of tubulointerstitial fibrosis[24,25]. Thus, the enhanced expression of angiotensinogen in proximal tubular cells under diabetic conditions, which may correlate with urinary angiotensiongen levels, may cause the tubulointerstitial injury and, then, result in the decline in eGFR. Also the augmentation of urinary angiotensinogen is considered to lead to increased formation of AngII in the kidney[9]. Thus, the increase of

urinary angiotensinogen may contribute to the development and progression of hypertension, which may associate with renal dysfunction and the incidence of cardiovascular disease. In the present study, urinary angiotenisinogen levels correlated with systolic and diastolic blood pressure as well as a previous report[12].

In this study, the data of clinical parameters including angiotensinogen were collected only at baseline. Thus, the time-dependent changes in these parameters during the follow-up were not evaluated. Also, the information regarding the use of RAS inhibitors during the follow-up period was not included in this study. Previous studies reported that RAS inhibitors were associated with reduction in urinary angiotensinogen levels[12,26]. In the present study, the levels of urinary angiotensinogen in patients treated with RAS inhibitors were not different from those without such treatment when data was analyzed separately according to the stage of nephropathy. In Japan, the prescription rate of RAS inhibitors in the past was much lower than that at present. Also, RAS inhibitors tended to be prescribed for patients who showed progression to the advanced stage of nephropathy or those at risk for cardiovascular disease. Thus, the present study does not provide conclusive data on the influence of RAS inhibitors on urinary angiotensinogen levels. Further studies are required to explore whether the reduction of urinary angiotensinogen level by any medication bring about improving renal and cardiovascular prognoses.

In conclusion, the present study demonstrated that urinary angiotensinogen levels correlated with progressive deterioration of renal function and the high incidence of renal-cardiovascular endpoints in patients with type 2 diabetes mellitus. These results suggest that higher levels of urinary angiotensinogen in patients with diabetic nephropathy are clinically useful to identify patients who are at high risk for worsening renal and cardiovascular complications. Also, the reduction of urinary angiotensinogen levels may be a new therapeutic index to prevent the worsening of renal and cardiovascular complications in diabetic patients with nephropathy.

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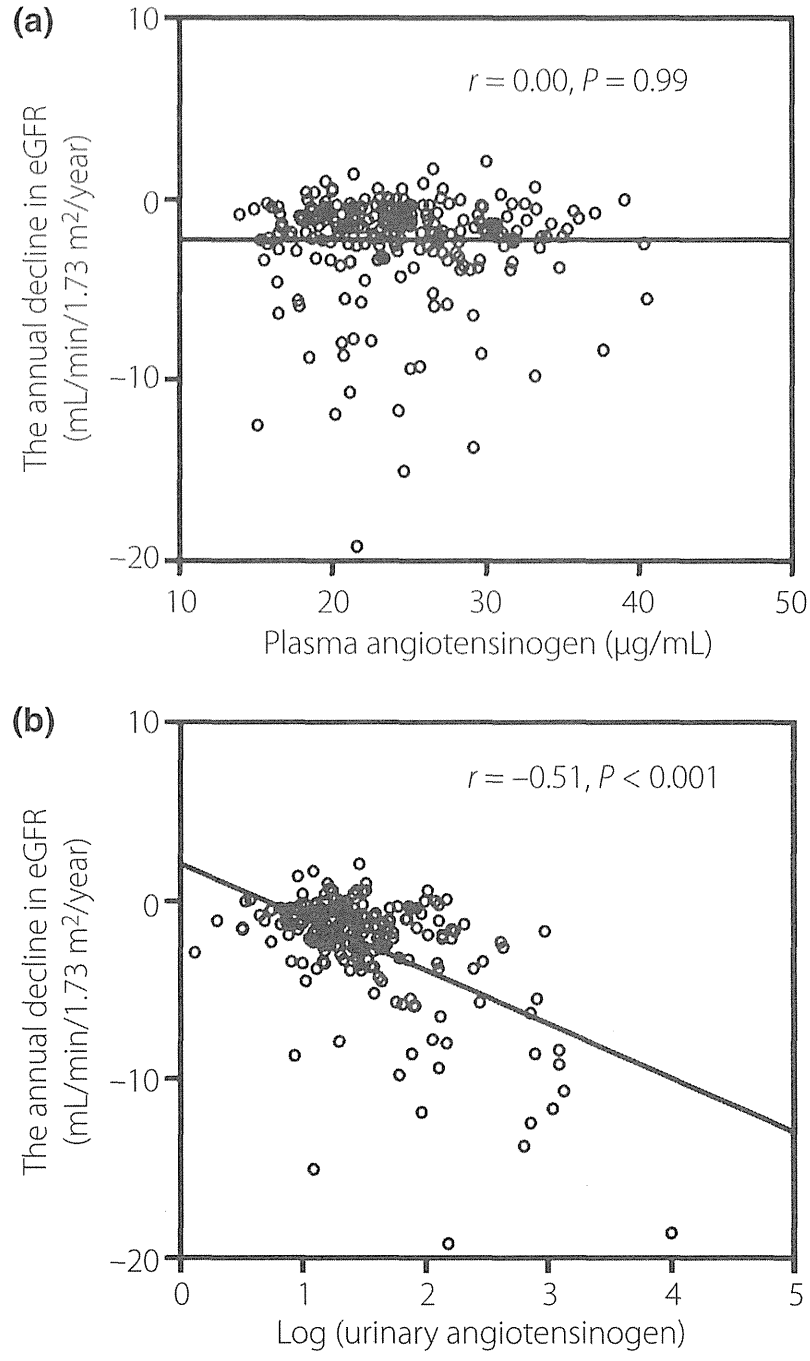

Figure 1.
Scatter diagram of the correlation between the annual decline in estimated glomerular filtration rate (eGFR) and (a) plasma angiotensinogen and (b) urinary angiotensinogen. Correlation was evaluated with the Pearson's correlation coefficient. Data are log-transformed values of urinary angiotensinogen.

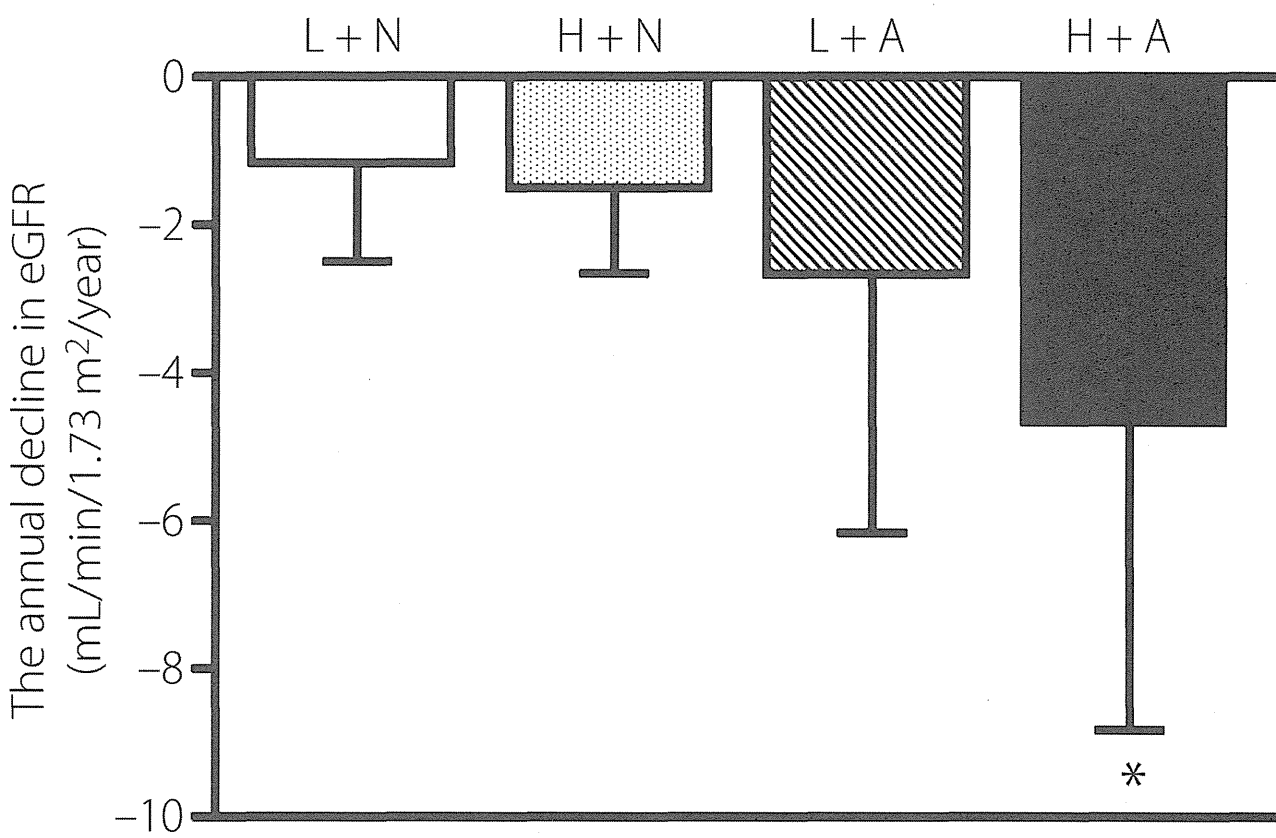

Figure 2.

Annual decline in estimated glomerular filtration rate (eGFR) during follow-up. Patients were divided into four groups using the median value of urinary angiotensinogen level (24.7 μg/g Cr) and the presence of albuminuria (>30 mg/g Cr). Patients with low levels of urinary angiotensinogen and normoalbuminuria (L + N, $n = 97$); patients with high levels of urinary angiotensinogen and normoalbuminuria (H + N, $n = 47$); patients with low levels of urinary angiotensinogen and albuminuria (L + A, $n = 21$) and patients with high levels of urinary angiotensinogen and albuminuria (H + A, $n = 69$). The respective annual decline in eGFR was: -1.2 ± 1.3, -1.4 ± 1.3, -2.7 ± 3.5 and -4.6 ± 4.2 mL/min/1.73 m^2/year. Data are mean ± SD. *$P < 0.05$ vs each other group (ANOVA with Tukey–Kramer HSD test).

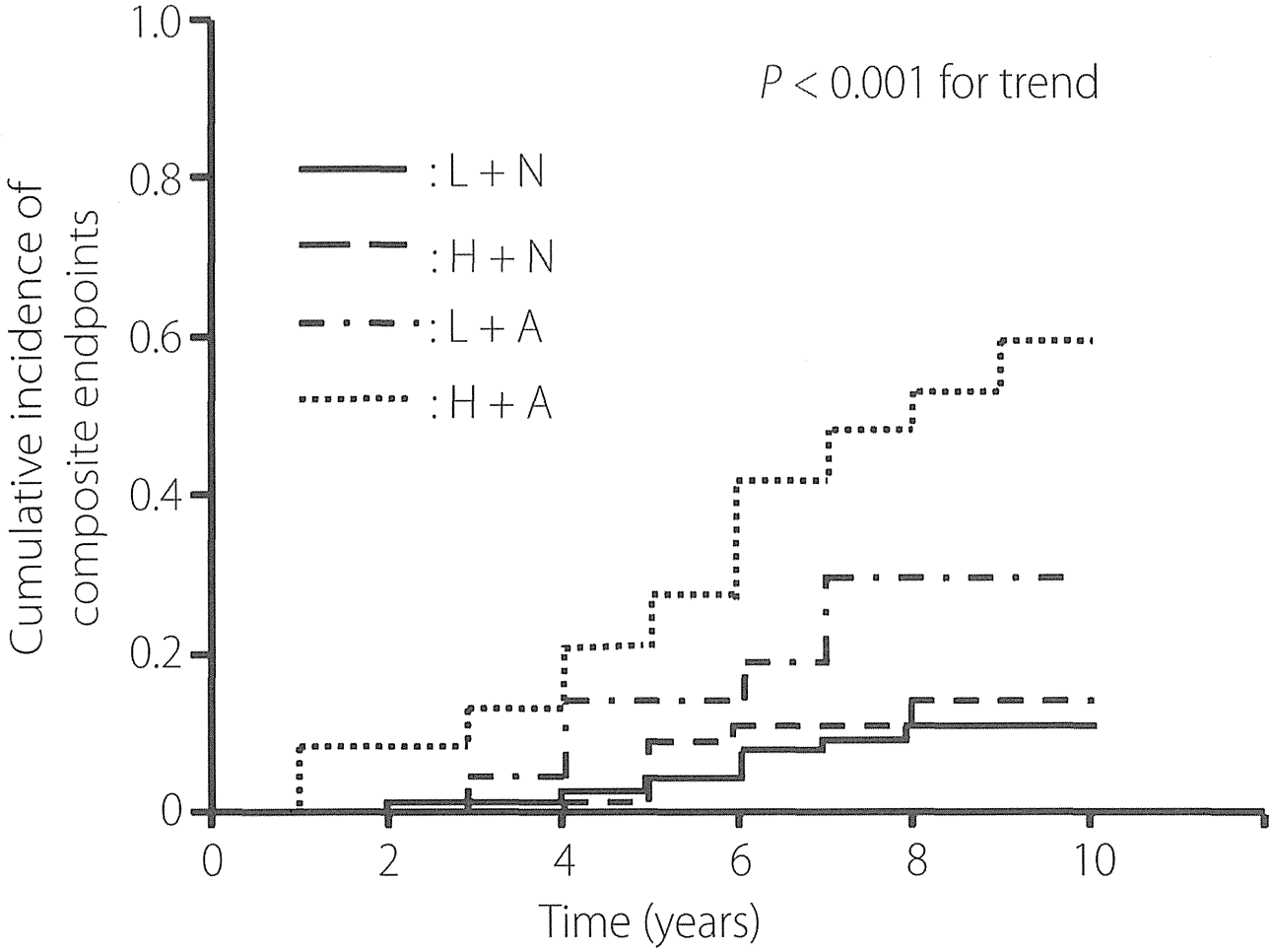

Figure 3.
Kaplan–Meier curves for cumulative incidence of renal and cardiovascular composite endpoints. Patients were divided into the four groups using the median value of urinary angiotensinogen level (24.7 μg/g Cr) and the presence of albuminuria (>30 mg/g Cr). Patients with low levels of urinary angiotensinogen and normoalbuminuria (L + N, $n = 97$); patients with high levels of urinary angiotensinogen and normoalbuminuria (H + N, $n = 47$); patients with low levels of urinary angiotensinogen and albuminuria (L + A, $n = 21$) and patients with high levels of urinary angiotensinogen and albuminuria (H + A, $n = 69$). Difference among the groups was tested by log rank test.

Table 1

Clinical characteristics of the study subjects

	Normoalbuminuria	Albuminuria	*P*
Number	144	90	
Gender (male/female)	72/72	57/33	<0.05
Age (year)	60 ± 8	59 ± 9	n.s.
Duration of diabetes (year)	13 ± 8	16 ± 8	<0.01
Body mass index (kg/m^2)	23.1 ± 3.4	24.5 ± 3.7	<0.01
Waist to hip ratio	0.93 ± 0.08	0.95 ± 0.09	n.s.
HbA1c (%)	7.4 ± 0.8	7.9 ± 1.2	<0.01
Systolic blood pressure (mmHg)	135 ± 17	144 ± 19	<0.01
Diastolic blood pressure (mmHg)	77 ± 9	81 ± 10	<0.01
Taking RAS inhibitors (%)	16	31	<0.01
Past history of CVD (%)	13	20	n.s.
Total cholesterol (mg/dL)	213 ± 32	219 ± 37	n.s.
HDL-cholesterol (mg/dL)	60 ± 156	56 ± 15	n.s.
Triglycerides (mg/dL)	111 ± 32	135 ± 76	<0.05
Urinary ACR (mg/g Cr)	10 (7–15)	161 (61–672)	<0.05
Estimated GFR (mL/min/1.73 m^2)	81 ± 15	69 ± 26	<0.01
Urinary β_2-microglobulin (μg/g Cr)	114 (73–172)	188 (81–907)	<0.01

Data are mean ± SD for normally distributed continuous variables or median (25th–75th interquartiles) for skewed continuous variables. Albuminuria represents microalbuminuria and overt proteinuria.

RAS, renin-angiotensin system; ACR, albumin-creatinine ratio; Cr, creatinine; GFR, glomerular filtration rate; CVD, cardiovascular disease.

Table 2

Factors that correlated with plasma and urinary angiotensinogen levels in univariate analysis

Parameter	Plasma angiotensinogen		Urinary angiotensinogen	
	r	*P*	*r*	*P*
Age	−0.02	0.75	−0.07	0.31
Duration of diabetes	−0.10	0.11	0.24	<0.001
Body mass index	0.17	0.009	0.13	0.04
Waist to hip ratio	0.36	<0.001	0.10	0.15
HbA1c	0.10	0.14	0.26	<0.001
Systolic blood pressure	0.14	0.03	0.30	<0.001
Diastolic blood pressure	0.10	0.13	0.23	<0.001
Total cholesterol	0.35	<0.001	0.17	0.008
HDL-cholesterol	0.20	0.002	−0.12	0.06
Triglycerides	0.18	0.006	0.19	0.004
Urinary ACR	0.01	0.88	0.77	<0.001
Estimated GFR	0.04	0.59	−0.44	<0.001
Urinary β_2-microglobulin	−0.07	0.26	0.72	<0.001

Correlation was evaluated with the Pearson's correlation coefficient.
The values of urinary angiotensinogen, urinary ACR and urinary β_2-microglobulin were log-transformed for the analysis because of their skewed distribution.
ACR, albumin-creatinine ratio; GFR, glomerular filtration rate.

Cholecystokinin Plays a Novel Protective Role in Diabetic Kidney Through Anti-inflammatory Actions on Macrophage

Anti-inflammatory Effect of Cholecystokinin

Satoshi Miyamoto,[1] Kenichi Shikata,[1,2] Kyoko Miyasaka,[3] Shinichi Okada,[1] Motofumi Sasaki,[1] Ryo Kodera,[1,2] Daisho Hirota,[1] Nobuo Kajitani,[1] Tetsuharu Takatsuka,[1] Hitomi Usui Kataoka,[1,4] Shingo Nishishita,[1] Chikage Sato,[1,5] Akihiro Funakoshi,[6] Hisakazu Nishimori,[7] Haruhito Adam Uchida,[1] Daisuke Ogawa,[1,5] and Hirofumi Makino[1]

Inflammatory process is involved in the pathogenesis of diabetic nephropathy. In this article, we show that cholecystokinin (CCK) is expressed in the kidney and exerts renoprotective effects through its anti-inflammatory actions. DNA microarray showed that CCK was upregulated in the kidney of diabetic wild-type (WT) mice but not in diabetic intracellular adhesion molecule-1 knockout mice. We induced diabetes in CCK-1 receptor (CCK-1R) and CCK-2R double-knockout (CCK-1R$^{-/-}$,-2R$^{-/-}$) mice, and furthermore, we performed a bone marrow transplantation study using CCK-1R$^{-/-}$ mice to determine the role of CCK-1R on macrophages in the diabetic kidney. Diabetic CCK-1R$^{-/-}$,-2R$^{-/-}$ mice revealed enhanced albuminuria and inflammation in the kidney compared with diabetic WT mice. In addition, diabetic WT mice with CCK-1R$^{-/-}$ bone marrow–derived cells developed more albuminuria than diabetic CCK-1R$^{-/-}$ mice with WT bone marrow–derived cells. Administration of sulfated cholecystokinin octapeptide (CCK-8S) ameliorated albuminuria, podocyte loss, expression of proinflammatory genes, and infiltration of macrophages in the kidneys of diabetic rats. Furthermore, CCK-8S inhibited both expression of tumor necrosis factor-α and chemotaxis in cultured THP-1 cells. These results suggest that CCK suppresses the activation of macrophage and expression of proinflammatory genes in diabetic kidney. Our findings may provide a novel strategy of therapy for the early stage of diabetic nephropathy. ***Diabetes* 61:897–907, 2012**

From the [1]Department of Medicine and Clinical Science, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan; the [2]Center for Innovative Clinical Medicine, Okayama University Hospital, Okayama, Japan; the [3]Department of Nutrition and Physiology, Tokyo Kasei University, Tokyo, Japan; the [4]Department of Primary Care and Medical Education, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan; the [5]Department of Diabetic Nephropathy, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan; the [6]Department of Gastroenterology, National Kyushu Cancer Center, Fukuoka, Japan; and the [7]Department of Hematology and Oncology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan.

Corresponding author: Kenichi Shikata, shikata@md.okayama-u.ac.jp.

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As the incidence of diabetes continues to increase in almost all areas of developing and developed countries, diabetic nephropathy has become the most common cause of end-stage renal disease worldwide (1). In addition, accumulating evidence suggests that the development of diabetic nephropathy leads directly to increased cardiovascular mortality (2). Recent studies have suggested that the inflammatory process plays a crucial role in the pathogenesis of diabetic nephropathy (3).

We previously focused on the relationship between intracellular adhesion molecule-1 (ICAM-1) expression and macrophage infiltration in the diabetic kidney. We reported that ICAM-1 was overexpressed on endothelial cells and mediated macrophage infiltration in the diabetic kidney (4). Furthermore, we demonstrated that blockade of macrophage infiltration using anti–ICAM-1 antibody (4) or ICAM-1 knockout (ICAM-1$^{-/-}$) mice (5) ameliorated diabetic renal injury, suggesting that the inflammatory axis of ICAM-1 activation to macrophage infiltration plays a pivotal role in the development of diabetic nephropathy.

In the current study, we performed a comprehensive, microarray-based analysis to clarify the genes responsible for the difference in urinary albumin excretion between diabetic ICAM-1$^{-/-}$ mice and diabetic wild-type (WT) mice. Unexpectedly, we found that cholecystokinin (CCK) mRNA expression was increased in the diabetic kidney of WT mice, whereas no significant increase was observed in nondiabetic WT mice.

CCK is a peptide hormone discovered in the small intestine (6,7) and is secreted from endocrine I cells of the duodenum and the jejunum into the bloodstream after a meal (8). CCK is well known as a regulator in the digestive tract and as a neurotransmitter in the nervous system (9,10). In addition to these well-known effects of CCK, anti-inflammatory effects of CCK have been reported (11–14).

To examine the role of CCK in the pathogenesis of diabetic nephropathy, diabetes was induced in CCK-1 receptor (CCK-1R) and CCK-2 receptor (CCK-2R) double-knockout (CCK-1R$^{-/-}$,-2R$^{-/-}$) mice. It is noteworthy that diabetic CCK-1R$^{-/-}$,-2R$^{-/-}$ mice exhibited increased albuminuria and showed increased levels of proinflammatory genes in the kidney cortex. Therefore, we speculated CCK had renoprotective effects, and we further examined the effects of