Table 1. Clinical profiles of enrolled subjects.

	Men			Women		
	Mean ± SD	Minimum	Maximum	Mean ± SD	Minimum	Maximum
Number of subjects	721			1063		
Age	47.9 ± 15.1	20	78	44.7 ± 13.9	20	79
Height (cm)	169.7 ± 6.0	143.7	186.7	156.9 ± 5.3	140.4	172.9
Body weight (kg)	71.3 ± 11.8	39.1	146.5	55.8 ± 9.6	29.3	118.0
Body mass index (kg/m^2)	24.7 ± 3.7	13.6	43.1	22.7 ± 3.8	14.1	44.9
Abdominal circumference (cm)	86.5 ± 10.3	62.4	135.0	78.3 ± 10.9	55.1	127.0
Right grip strength (kg)	42.4 ± 7.7	3.4	70.2	25.6 ± 5.1	7.1	45.1
Left grip strength (kg)	40.4 ± 7.6	4.6	63.1	24.3 ± 4.9	4.5	43.5
Leg strength (kg)	67.1 ± 17.5	19.0	140.0	41.5 ± 11.2	11.0	79.0
Leg strength per body weight	0.95 ± 0.22	0.28	1.65	0.75 ± 0.19	0.17	1.46

The study was approved by the Ethics Committee of Okayama Health Foundation.

2.2. Anthropometric Measurements

The anthropometric parameters were evaluated by using the following respective parameters such as height, body weight, body mass index (BMI), abdominal circumference, and hip circumference. BMI was calculated by weight/[height]^2 (kg/m^2). The abdominal circumference was measured at the umbilical level in standing subjects after normal expiration [11].

2.3. Muscle Strength

To assess muscle strength, grip and leg strength were measured [12]. Grip strength was measured using THP-10 (SAKAI, Tokyo, Japan), while leg strength was measured by COMBIT CB-1 (MINATO, Osaka, Japan). Isometric leg strength was measured as follows: the subject sat in a chair, grasping the armrest in order to fix the body position. A dynamometer was then attached to the subject's one ankle joint by a strap. The subject extended his or her leg to 60 degrees as described in previous reports [12,13] which have also demonstrated good accuracy for this measurement [13]. All muscle strength measurements were recorded in 2 trials, and the better one was employed for analysis. In addition, to standardize the influence of body weight, we calculated the ratio of leg strength to body weight; a ratio of 1.0 in leg strength per body weight has been a standard in past studies [13].

2.4. Urine Examination

Urine samples were collected from the second-morning urine (before 10 a.m.) and examined within 1 hour. The urine examination was performed using urine strip tests (BAYER, Tokyo, Japan). The reagent strip was dipped directly into the urine sample. Just after dipping, the sample is graded as –: negative, ±: trace positive, +: positive (30 mg/dl), 2+: positive (100 mg/dl), 3+: positive (300 mg/dl) or 4+: positive (1000 mg/dl) by comparison with a standard color chart found on the container's label [14].

2.5. Statistical Analysis

All data are expressed as mean ± standard deviation (SD) values. A statistical analysis was performed using an unpaired t test and covariance analysis, where $p < 0.05$ was considered to be statistically significant.

3. RESULTS

Clinical profiles are summarized in **Table 1**. Leg strength was 67.1 ± 17.5 kg in men and 41.5 ± 11.2 in women. Prevalence of proteinuria in enrolled subjects is also summarized in **Table 2**. A total of 35 men (4.9%) and 27 women (2.5%) was diagnosed as having the proteinuria (+: 30 mg/dl ≦).

We compared muscle strength between subjects with and without proteinuria (**Table 3**). In men, leg strength and leg strength per body weight in subjects with proteinuria was significantly lower than those in subjects without proteinuria even after adjusting for age by using covariance analysis (leg strength: $p = 0.0017$, leg strength per body weight: $p = 0.0495$). The significant differences of grip strength were not noted in men at a significant level (right grip strength: $p = 0.3691$, left grip strength: $p = 0.0670$). In women, parameters of muscle strength in subjects with proteinuria were not significant different from those in subjects without proteinuria (**Table 3**).

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Table 2. Prevalence of proteinuria in enrolled subjects.

Proteinuria	20's	30's	40's	50's	60's	70's	Total	%
Men								
−	72	120	132	138	124	25	611	84.7
±	7	18	13	12	18	7	75	10.4
+	4	3	3	6	4	3	23	3.2
2+	0	3	2	1	3	1	10	1.4
3+	0	0	0	0	1	0	1	0.1
4+	0	0	0	0	1	0	1	0.1
Total	83	144	150	157	151	36	721	
Women								
−	165	224	202	207	144	30	972	91.4
±	13	15	10	18	8	0	64	6.0
+	5	1	3	5	2	0	16	1.5
2+	2	1	3	0	0	2	8	0.8
3+	1	2	0	0	0	0	3	0.3
Total	186	243	218	230	154	32	1063	

Table 3. Comparison of muscle strength between subjects with and without proteinuria.

	Proteinuria (− or ±)	Proteinuria (+≦)	p	p After adjusting for age
Men				
Number of subjects	686	35		
Age	47.8 ± 14.1	51.3 ± 16.2	0.1553	
Right grip strength (kg)	42.6 ± 7.6	39.6 ± 9.9	**0.0284**	0.3691
Left grip strength (kg)	40.5 ± 7.5	37.8 ± 8.9	**0.0379**	0.0670
Leg strength (kg)	67.3 ± 17.2	62.9 ± 21.7	0.1509	**0.0017**
Leg strength per body weight	0.95 ± 0.22	0.83 ± 0.26	**0.0017**	**0.0495**
Women				
Number of subjects	1036	27		
Age	44.8 ± 13.9	42.3 ± 16.3	0.3519	
Right grip strength (kg)	25.7 ± 5.1	23.5 ± 5.0	**0.0294**	0.7149
Left grip strength (kg)	24.3 ± 4.9	22.7 ± 4.4	0.0877	0.6094
Leg strength (kg)	41.5 ± 11.2	40.9 ± 11.5	0.7804	0.4926
Leg strength per body weight	0.75 ± 0.19	0.71 ± 0.18	0.2672	0.8468

4. DISCUSSION

In this study, we firstly evaluated the link between proteinuria and muscle strength *i.e.* grip strength, leg strength and leg strength per body weight in Japanese. Proteinuria might be a modifiable factor of muscle strength, especially in Japanese men.

Proteinuria and/or reduced renal function have been reported to be closely linked to cardio vascular disease (CVD) [15,16]. Anavekar *et al.* showed that even mild renal disease was considered a major risk factor for CVD after myocardial infarction in 14527 patients with acute myocardial infarction [15]. Irie *et al.* reported that they evaluated 30,764 men and 60,668 women aged 40 - 79 years for 10 years, and proteinuria and hypercreati-nemia or reduced GFR and their combination were sig-

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nificant predictors of CVD and all-cause mortality [16]. We have also reported that proteinuria was a modifiable factor for cardiorespilatory fitness evaluated by VT [6]. However, according to the link between proteinuria and muscle strength, there were few studies especially in Japan. Protein-energy wasting is the term proposed to describe the reduction in the stores of energy and protein in patients CKD [17]. Muscle wasting is one of the best markers of protein-energy wasting in these patients [18]. Leal *et al.* reported that handgrip strength is a useful tool for continuous and systematic assessment of muscle mass related to nutritional status in patients on dialysis [19]. Takhreen reviewed that relationship between exercise intervention and quality of life (QOL) in CKD patients. Exercising patients have shown improvements in physical fitness, psychological function, reaction times and lower extremity muscle strength, and these factors help improve QOL [20]. In this study, we solely evaluated the relationship between proteinuria and muscle strength *i.e.* grip strength, leg strength and leg strength per body weight in the Japanese. The significant differences of leg strength and leg strength per body weight between men with and without proteinuria even after adjusting for age. However, muscle strength in women with proteinuria was not significantly lower than that in women without.

Potential limitations still remain in this study. First, our study was a cross sectional and not a longitudinal study. Second, 721 men and 1063 women in our study voluntarily underwent measurements: they were therefore more likely to be health-conscious compared with the average person. Second, we could not show clear mechanism between proteinuria and muscle strength. We have previously reported that brachial-ankle pulse wave velocity (baPWV) in subjects with reduced eGFR was significantly higher than that in subjects without [21]. In addition to protein-energy wasting, arterial stiffness might affect the results. Third, significant difference of muscle strength was not noted in women in this study. Low prevalence of proteinuria also affected the results, especially in women. To show this, further prospective studies are needed in the Japanese.

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REVIEW ARTICLE

Clinical impact of albuminuria in diabetic nephropathy

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Abstract Patients suffering from diabetic nephropathy, resulting in end-stage renal failure, are increasing in number. The pathophysiology of diabetic nephropathy remains to be fully investigated. In the clinical setting, the presence of albuminuria/overt proteinuria and a low glomerular filtration rate may predict poor renal prognosis, but the prognosis of the normoalbuminuric renally insufficient diabetic patient remains controversial. In addition to the measurement of urinary albumin excretion, biomarker studies to detect diabetic nephropathy more specifically at the early stage have been performed worldwide. There is a growing body of evidence for remission and/or regression of diabetic nephropathy, which may be an indicator for cardiovascular and renal risk reduction. Deeper insights into the pathological characteristics as well as the clinical impact of albuminuria on renal and cardiovascular outcome are required.

Keywords Diabetic nephropathy · Albuminuria · Proteinuria · Glomerular filtration rate · Cardiovascular disease · Renal outcome

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Introduction

Based on the annual report of the Japanese Society for Dialysis Therapy (JSDT), diabetic nephropathy is a leading cause of end-stage renal failure in Japan [1]. The number of dialysis patients had increased to 297,126 by the end of 2010. According to the annual report of the JSDT, diabetic nephropathy has been a leading primary disease of new patients who have been started on dialysis since 1998 [1]: the number of such patients with diabetic nephropathy has increased to 43.5%. In addition, cardiovascular diseases and deaths in patients with diabetes and underlying renal disease before and after dialysis has increased [2, 3]. Therefore, preventing and halting the progression of diabetic nephropathy is important if we are to prolong the survival of such patients.

Characteristic pathologic changes associated with diabetic nephropathy are accumulation of extracellular matrix (ECM) and the infiltration of inflammatory cells into glomeruli and tubulointerstitial regions [4, 5]. These pathologic abnormalities are induced by alterations in ECM production or degradation [6]. Generally speaking, the occurrence of albuminuria is a reflection of increased matrix deposition, leading to glomerular and tubulointerstitial lesions. Diabetic nephropathy is a clinical entity in

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which the presence of persistent albuminuria and declines in renal function and glomerular filtration rate (GFR) are the major characteristic findings, which are closely associated with end-stage renal diseases, enhanced cardiovascular morbidity and eventual mortality [7]. The incidence of albuminuria, which currently contributes to the diagnosis of diabetic nephropathy, is well correlated with a decrease in GFR and the incidence of cardiovascular diseases.

Here, we focus on the clinical impact of albuminuria along with GFR levels on the progression of diabetic nephropathy and the incidence of cardiovascular diseases, which is closely related to the mortality of patients with diabetic nephropathy in this manuscript.

Albuminuria in the diagnosis of diabetic nephropathy

The definitive diagnosis of diabetic nephropathy is based on pathological findings such as the presence of diffuse mesangial lesions and nodular lesions. However, renal biopsy is not performed for all patients with diabetic nephropathy. In the clinical setting, the presence of persistent proteinuria as well as other complications such as diabetic retinopathy and renal dysfunction is important in the diagnosis of diabetic nephropathy. However, early detection of the presence of diabetic nephropathy is clinically required for the best prognosis. The measurement of urinary albumin excretion is currently crucial to the detection of early diabetic nephropathy. The increased excretion of albumin (albuminuria) is an early diagnostic indicator of diabetic nephropathy. Thus, Mogensen et al. [8] proposed a classification of diabetic nephropathy in patients with type 1 diabetes based on increased urinary albumin excretion once diabetic nephropathy was diagnosed. Diabetic nephropathy is also staged in Japan [9, 10], and the staging was described by Yokoyama et al. [11] as follows: stage I: urinary albumin-to-creatinine ratio (ACR) <30 mg/g creatinine; stage II: ACR $\geq$ 30 and <300 mg/g creatinine (i.e., albuminuria); stage III: ACR $\geq$ 300 mg/g creatinine and/or persistent proteinuria with serum concentration of creatinine <2 mg/dl; stage IV: serum concentration of creatinine $\geq$2 mg/dl with proteinuria; and stage V: being treated with dialysis. The Japan Diabetes Clinical Data Management Study Group (JDDM) reported that the prevalence of albuminuria (stage II) in Japanese type 2 diabetic patients was 32%, which is very similar to the 39% observed in the DEMAND study [12]. These results suggest that albuminuria is common, and that 76% of patients with diabetic nephropathy are categorized as stage II, as evidenced by the presence of albuminuria. Further, 58% of the patients enrolled were at stage I, 7% were at stage III, 2.6% were at stage IV, and 0.4% were at stage V [11]. A very recent study from the Japan Diabetes Complications Study (JDCS) revealed that the annual transition rate to proteinuria (ACR $\geq$ 300 mg/g creatinine) was 0.67%, and that this was substantially higher for the low-albuminuric group (defined as a urinary ACR of 30–150 mg/g creatinine) than for the normoalbuminuric group (defined as a urinary ACR of <30 mg/g creatinine) [13]. In this sense, UKPDS 64 reported that the progression to albuminuria occurred at 2.0% per year, and from albuminuria to macroalbuminuria at 2.8% per year [14]. However, about 40% of the diabetic patients had no urinary albumin excretion measurements, regardless of the recommendation for the JDDM cohort [11]. Therefore, the measurement of urinary albumin excretion is required for the early detection of diabetic nephropathy in Japan.

Biomarkers for diabetic nephropathy and disease progression

Further studies to detect diabetic nephropathy more specifically at the early stage in addition to urinary albumin excretion are needed. In this sense, biomarker studies to identify the presence and predict the progression of diabetic nephropathy have been performed worldwide [15]. Recently, Kamijo-Ikemori et al. [16] reported that urinary levels of liver-type fatty acid-binding protein (L-FABP) accurately reflected the severity of diabetic nephropathy in type 2 diabetes. Importantly, urinary L-FABP levels were high in patients with normoalbuminuria, suggesting its usefulness for detecting early nephropathy in these patients. Further, an increase in urinary Smad1—a key transcriptional factor for mesangial matrix expansion in diabetic nephropathy—at the early stage was correlated with subsequent development of glomerulosclerosis in experimental rodent models [17]. Regarding renal function, serum cystatin C was reported to be a good marker for nephropathy [18]. Notably, cases of early renal dysfunction, defined by a loss of cystatin C GFR exceeding −3.3%/year, occurred in 9% of the normoalbuminuria group and 31% of the albuminuria group [19].

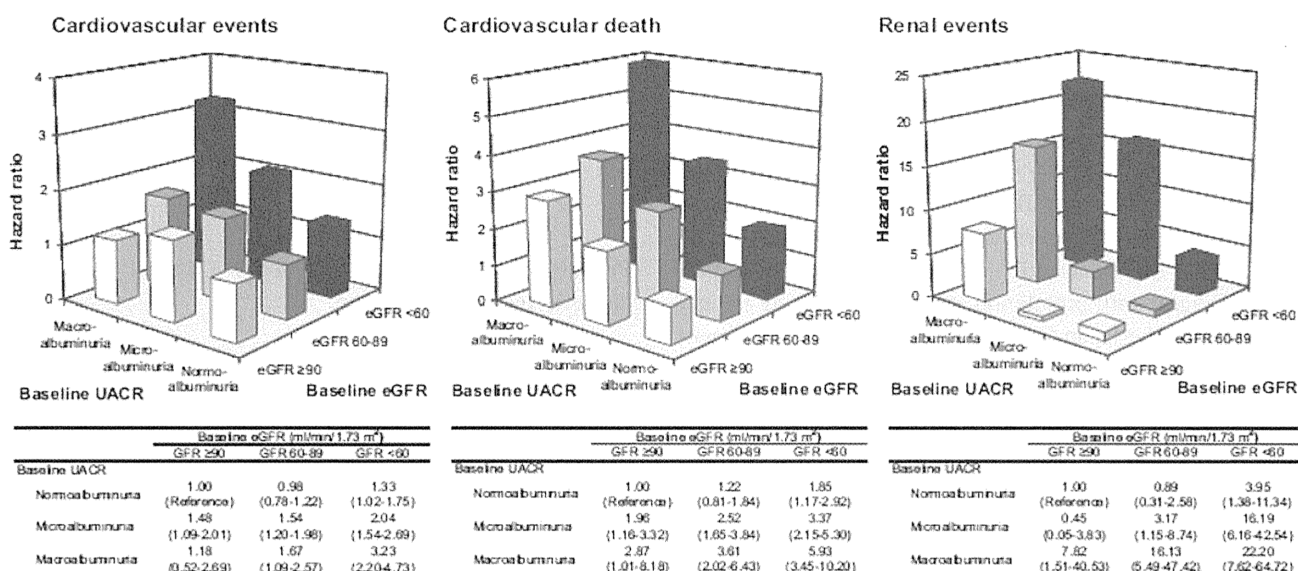
Prevalence of albuminuria and low GFR in type 2 diabetic patients in Japan

As previously described, diabetic nephropathy is diagnosed through the detection of albuminuria. Recently, Kidney Disease: Improving Global Outcomes (KDIGO) reported the definition, classification and prognosis of chronic kidney disease based on both estimated GFR and urinary levels of albumin excretion [20]. In this sense, there are diabetic patients with decreases in GFR and normoalbuminuria. Is diabetic nephropathy observed in such patients? In fact, the percentage of diabetic patients with normoalbuminuria and low estimated GFR is believed to be relatively high. Importantly, Yokoyama et al. [21] described

that the proportion of subjects with low estimated GFR (<60 ml/min/1.73 m^2) and normoalbuminuria was 11.4% of the type 2 diabetic patients examined (262/2298). In this manuscript, 63.4% of the 262 patients studied had neither diabetic retinopathy nor neuropathy. On the other hand, these patients were older and included a higher proportion of women and patients with hypertension, hyperlipidemia and cardiovascular disease, as well as fewer smokers compared with those with normoalbuminuria and preserved GFR. In contrast, the proportion of type 2 diabetic patients with preserved GFR but albuminuria or overt proteinuria was 27% (755/2791). Most importantly, the lack of histologically proven diabetic nephropathy should be discussed. In type 1 diabetes patients with normoalbuminuria and low GFR, renal biopsy specimens revealed more advanced diabetic glomerular lesions. It is worth noting that a reduced GFR was found much more often among female patients, particularly if retinopathy and/or hypertension were also present [22]. Deep insight into the prevalence and prognoses of these patients with proven pathological characteristics and grading is required to understand the pathophysiology of diabetic nephropathy in greater depth, together with future perspectives.

Clinical impacts of albuminuria and GFR on the prognoses of diabetic patients

Obviously, diabetic patients who had both albuminuria/overt proteinuria and low GFR were at risk of adverse outcomes, including cardiovascular events, cardiovascular death, and renal events, as reported by the Action in Diabetes and Vascular Disease: Preterax and DiamicroN MR Controlled Evaluation (ADVANCE) study [23] (Fig. 1). Do normoalbuminuric renally insufficient diabetic patients have a poor prognosis? Rigalleau et al. [24] reported that the risks of renal progression and death in these patients with type 1 or type 2 diabetes are lower. Concomitantly, in type 2 diabetic patients, the Casale Monferrato study revealed that macroalbuminuira was the main predictor of mortality, independently of both estimated GFR and cardiovascular risk factors, whereas the estimated GFR provided no further information on all-cause mortality and cardiovascular mortality in normoalbuminuric patients [25]. Supporting this notion, regarding renal end-points, there was also a progressive increase in risk associated with declined renal function, which was mainly observed in the albuminuric group in Chinese type 2 diabetic patients [26]. Interestingly, those with a reduced estimated GFR were at high risk of developing cardiovascular end-points (cardiovascular death, new admissions due to angina, myocardial infarction, stroke, revascularization or heart failure) and all-cause mortality, independent of albuminuria [26]. In contrast, as previously described, in the ADVANCE study, patients with normoalbuminuria and estimated GFR <60 ml/min per 1.73 m^2 had a 3.95-fold higher risk of renal events, a 1.33-fold higher risk of cardiovascular events, and a 1.85-fold higher risk of cardiovascular death [23] (Fig. 1). Moreover, Vlek et al. reported that an estimated GFR <60 ml/min/1.73 m^2 without albuminuria was



Cardiovascular events

	Baseline eGFR (ml/min/1.73 m^2)		
	GFR ≥90	GFR 60-89	GFR <60
Baseline UACR			
Normoalbuminuria	1.00 (Reference)	0.98 (0.78-1.22)	1.33 (1.02-1.75)
Microalbuminuria	1.48 (1.09-2.01)	1.54 (1.20-1.98)	2.04 (1.54-2.69)
Macroalbuminuria	1.18 (0.52-2.69)	1.67 (1.09-2.57)	3.23 (2.20-4.73)

Cardiovascular death

	Baseline eGFR (ml/min/1.73 m^2)		
	GFR ≥90	GFR 60-89	GFR <60
Baseline UACR			
Normoalbuminuria	1.00 (Reference)	1.22 (0.81-1.84)	1.85 (1.17-2.92)
Microalbuminuria	1.96 (1.16-3.32)	2.52 (1.65-3.84)	3.37 (2.15-5.30)
Macroalbuminuria	2.87 (1.01-8.18)	3.61 (2.02-6.43)	5.93 (3.45-10.20)

Renal events

	Baseline eGFR (ml/min/1.73 m^2)		
	GFR ≥90	GFR 60-89	GFR <60
Baseline UACR			
Normoalbuminuria	1.00 (Reference)	0.89 (0.31-2.58)	3.95 (1.38-11.34)
Microalbuminuria	0.45 (0.05-3.83)	3.17 (1.15-8.74)	16.19 (6.16-42.54)
Macroalbuminuria	7.82 (1.51-40.53)	16.13 (5.49-47.42)	22.20 (7.62-64.72)

Fig. 1 Combined effects of albuminuria and eGFR levels at baseline on the risk of an adverse outcome. The estimates are adjusted for baseline covariates, including age, gender, duration of diabetes, SBP, history of currently treated hypertension, history of macrovascular disease, HbA1c, LDL cholesterol, HDL cholesterol, log-transformed triglycerides, BMI, electrocardiogram abnormalities, current smoking, and current drinking. (From Ref. [23] reproduced with permission from the American Society of Nephrology)

the strongest risk factor in the occurrence of vascular events (hazard ratio 1.50; 1.05–2.15) [27]. Recently, the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study revealed that normoalbuminuric patients with eGFR 30–59 ml/min per 1.73 m^2 were at higher risk of a cardiovascular event, cardiovascular death, noncoronary heart disease death, and death from any cause than normoalbuminuric patients with eGFR $\geq$60 ml/min per 1.73 m^2 [28]. Interestingly, high normal levels of albuminuria ($\geq$5 μg/min) predicted the development of micro- and macroalbuminuria and increased mortality in Brazilian type 2 diabetic patients [29]. Furthermore, in Japanese patients with type 1 and type 2 diabetes, even within the normal range ($\leq$30 mg/g), ACR $\geq$10 mg/g in women and $\geq$5 mg/g in men was associated with a significantly greater rate of decline in eGFR relative to subjects with ACR $\leq$5 mg/g [30]. It is of interest that the risk of cardiovascular events in individuals with diabetes increases with the ACR, starting well below the microalbumin cutoff [31]. Taken together, an evaluation of the clinical impact of albuminuria along with an evaluation of the effect of GFR on the prognoses of diabetic patients is required.

Remission/regression of albuminuria in patients with diabetic nephropathy

Fioretto et al. [32] reported that pancreas transplantation reversed the lesions of diabetic nephropathy in patients with type 1 diabetes mellitus, but that reversal required more than 5 years of normoglycemia. A growing body of evidence since then has pointed to the possibility of remission and/or regression of diabetic nephropathy, especially in patients treated with renin-angiotensin system blockade drugs. However, there is a lack of data on pathological findings in these patients. In the clinical setting, Perkins et al. [33] stated that regression of albuminuria was frequent in patients with type 1 diabetes mellitus, with a 6-year cumulative incidence of 58%. In this context, the definition of regression of microalbuminuria is a 50% reduction in albumin excretion from one 2-year period to the next. In addition, Hovind et al. [34] at the Steno Diabetes Center reported that the total number of patients who obtained remission was 92 (31%), with a duration of remission of 3.4 years, and regression occurred in 67 (22%) of 301 consecutive type 1 diabetic patients with diabetic nephropathy. Remission was defined as albuminuria <200 μg/min sustained for at least 1 year and a decrease of at least 30% from pre-remission levels, and regression as a rate of decline in GFR equal to the natural aging process: $\leq$1 ml/min/year during the investigation period in this report. Moreover, remission of nephrotic-range albuminuria in type 1 diabetic patients was also reported at the Steno Diabetes Center [35]. In this report, remission was induced in 28 of 126 (22%) patients; 21 were predominantly treated with angiotensin-converting enzyme (ACE) inhibitors, and 7 with non-ACE inhibitor medications. Remission lasted 3.6 years. In particular, more women (37%) than men (16%) obtained remission. In addition to type 1 diabetic patients, recent studies have revealed that remission is induced in type 2 diabetic patients. Araki et al. [36] reported that a reduction in urinary albumin excretion rate was frequent, with a 6-year cumulative incidence of 51% for remission, defined as a shift to normoalbuminuria, and 54% for regression, defined as a 50% reduction in the urinary albumin excretion rate. Interestingly, in this particular study, the frequency of progression to overt proteinuria was 28%, and albuminuria of short duration, the use of renin-angiotensin system-blocking drugs, and lower titers for HbA1c and systolic blood pressure were independently associated with remission or regression. More recently, JDCS revealed that a return from low microalbuminuria to normoalbuminuria was observed in 137 out of 452 patients (30.3%) [13].

Further, the clinical impact of remission/regression on renal outcome and cardiovascular events is still to be fully investigated. Importantly, Araki et al. [37] have reported that a reduction in albuminuria in patients with type 2 diabetes is an indicator of cardiovascular and renal risk reduction. In this study, the cumulative incidence of mortality from and hospitalization for renal and cardiovascular events was significantly lower in patients with a 50% reduction. Collectively, remission/regression in patients with diabetic nephropathy is relatively frequent, and insight into the pathological characteristics as well as the clinical impact on renal and cardiovascular outcomes when remission/regression is induced is needed.

Hematuria in diabetic nephropathy

Hematuria, the other major characteristic finding aside from albuminuria/overt proteinuria, was reported in 14 out of 34 Japanese patients with biopsy-proven diabetic nephropathy [38]. Patients with hematuria had significantly lower renal function, and the prevalences of nephrotic syndrome and retinopathy were significantly higher than in patients without hematuria. Interestingly, based on a logistic regression analysis, the presence of nephrotic syndrome and a known duration of diabetes were identified as significant predictors for hematuria with diabetic nephropathy.

Concluding remarks and future directions

Deep insights into the onset and progression of albuminuria along with GFR may elucidate the pathogenesis of

progressive kidney complications and associated cardiovascular diseases. Further studies of the clinical characteristics and the pathological findings of kidney involvement in patients with diabetes are required for a better understanding of diabetic nephropathy and the benefits of therapy for it.

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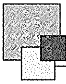

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Pathogenesis of diabetic complications through bone marrow-derived cells

Diabetic nephropathy is a major microvascular complication of diabetes mellitus. It is by far the most common cause of end-stage kidney failure worldwide. Recently, chronic kidney disease, CKD, which is defined by proteinuria or reduced kidney function, has been emerged. As kidney function deteriorates, development of cardiovascular disease is promoted, and the risk is much higher than that of the progression of end-stage kidney failure. Therefore, clarifying the mechanism of the progression of CKD including diabetic nephropathy is very important not only for preventing the progression of kidney diseases but also improving the survival of patients with CKD.

In the course of the progression of both glomerulonephritis and diabetic nephropathy, the accumulation of matrix proteins leading to glomerular sclerosis and interstitial fibrosis is a prominent feature of the disease *(Figure 1)*.[1,2] In this histopathological picture, the accumulation of bone marrow-derived cells such as monocytes/macrophages in the diseased kidneys is a hallmark of the progression of CKD (1). In addition to these pathological factors, proteinuria is clinically a critical factor in the progression of CKD including diabetic nephropathy.[3]

Recently, circulating mesenchymal progenitor cells, originally termed fibrocytes, have been shown to be involved in the pathogenesis of organ fibrosis (1). These cells comprise minor fraction of peripheral leukocytes in healthy humans. These cells have surface markers, CD45 and CD34, and produce extracellular matrix proteins such as type 1 collagen (Col1). Based on these characters, these distinct type of the cells have been originally identified by CD34 and Col1. Although a recent study revealed that markers (CD45RO, 25F9, S100A8/A9) distinguish monocyte-derived fibrocytes from monocytes, macrophages and fibroblasts, identification of markers specific to fibrocytes remains to be investigated. So far, identification of these cell populations may be identified by dual positivity of CD34 or CD45 and Col1 or pro-Col1 in most settings. In addition, these cells express chemokine receptors such as CCR2, CCR7, and CXCR4. Further, these cells have a potential to differentiate into myofibroblasts and adipocytes by stimulation with transforming growth factor (TGF)-ß1 and peroxisome-proliferator-activated receptor gamma, respectively. Physiologically, these cells are involved in the wound healing and maintenance of auditory function.

We previously identified these CD45 and Col1 dual-positive ($CD45^+/Col1^+$) cells in fibrotic kidney induced by ureteral ligation in mice.[4] Together with results from in vitro studies,

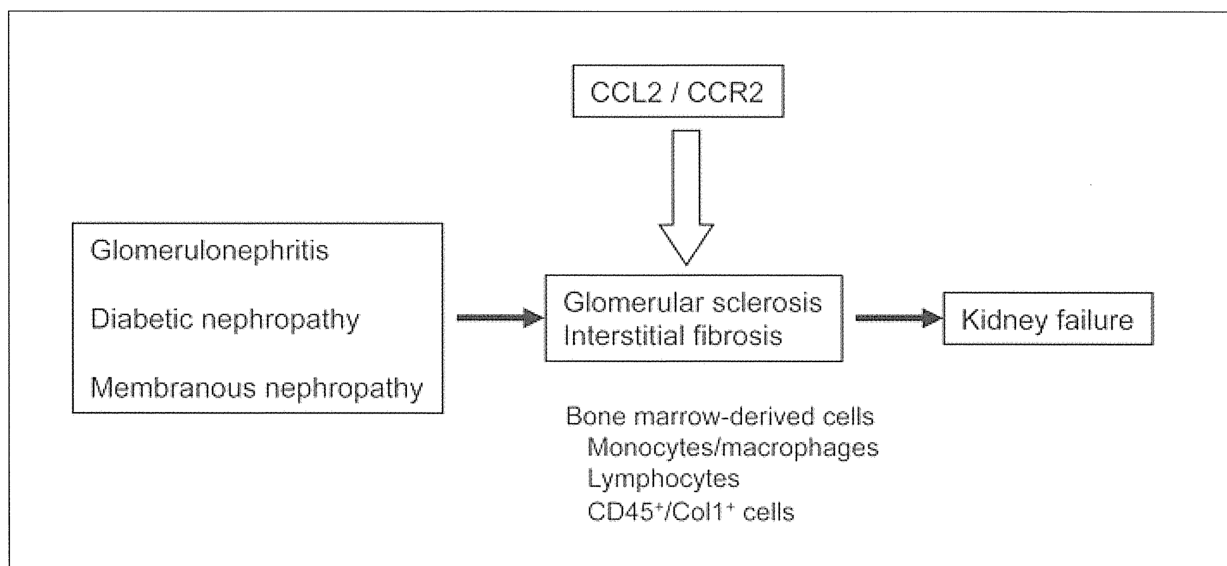
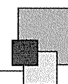


Figure 1. CCL2 / CCR2: The common regulatory factor in the progression of chronic kidney disease.



CD45+/Col1+ cells may contribute to kidney fibrosis by at least 3 mechanisms.[1] First, these cells produce CCL2 / monocyte chemoattractant protein (MCP)-1, a potent chemokine for macrophage recruitment and activation. Accumulating evidence has uncovered that CCL2 plays an important role through its cognate receptor, CCR2, and its downstream signal cascade in the pathogenesis of diabetic nephropathy as well as glomerulonephritis *(Figure 1)*.[1,5] Among these, a previous study showed that up-regulation of locally produced CCL2 might be involved in advanced tubulointerstitial lesions in diabetic patients with nephrotic syndrome through macrophage recruitment and activation.[6] Second, CD45+/Col1+ cells produce TGF-β1, resulting in stimulating fibroblasts to produce collagen. Third, these cells directly produce Col1.

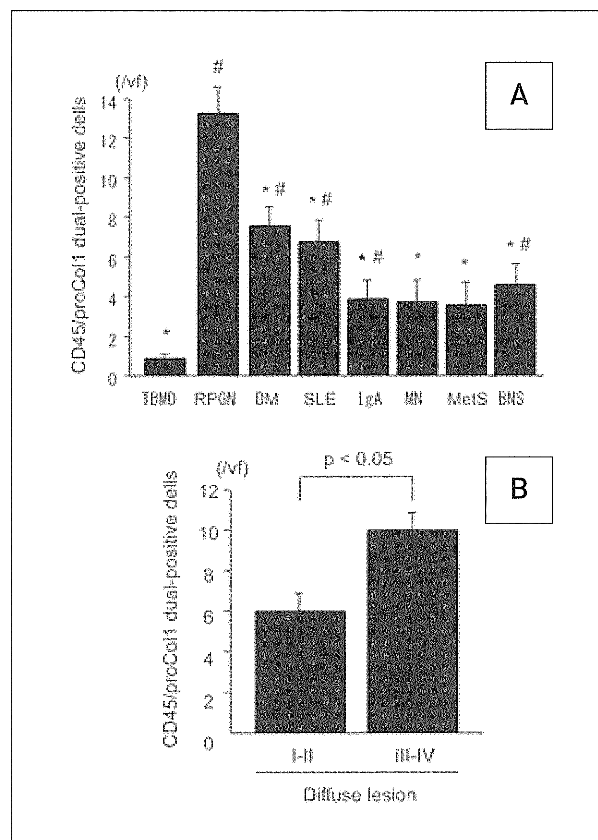


Figure 2. The number of CD45 and procollagen (proCol) 1 dual-positive cells in specimens of kidney in patients with chronic kidney disease. (A) TBMD, thin basement membrane disease; RPGN, rapidly progressive glomerulonephritis; DM, diabetes mellitus; SLE, systemic lupus erythematosus; IgA, IgA nephropathy; MN, membranous nephropathy; MetS, metabolic syndrome; BNS, benign nephrosclerosis. # $p < 0.05$ vs. TBMD, * $p < 0.05$ vs. RPGN. Bars indicate means±S.E.M. (B) Bars indicate means±S.E.M.

Following animal and in vitro studies, we examined the presence of CD45+/proCol1+ cells in 113 kidney specimens of human CKD including diabetic nephropathy.[7] CD45+/proCol1+ cells were mostly identified in the kidneys of patients with rapidly progressive glomerulonephritis among specimens investigated *(Figure 2A)*. Interestingly, CD45+/proCol1+ cells were detected in specimens of diabetic nephropathy. The number of CD45+/proCol1+ cells in the interstitium correlated with the extent of interstitial fibrosis, the number of CD68-positive macrophages, and urinary CCL2 levels in patients with CKD. As far as diabetic nephropathy was concerned, the number of interstitial CD45+/proCol1+ cells was larger in patients with advanced glomerular diffuse lesions (III-IV) than that in mild lesions (I-II) *(Figure 2B)*. Of note, there was an inverse correlation between the number of interstitial CD45+/Col1+ cells and kidney function at the time of biopsy. Taken together, these results suggest that CD45+/Col1+ cells may be involved in the progression of CKD through the interaction with macrophages and CCL2.

Based on these findings, we examined the involvement of CD45+/Col1+ cells in the progression of diabetic nephropathy. In vitro studies demonstrated that human CD45+/Col1+ cells under high glucose concentration expressed CCR2 and directly contributed to producing Col1 (unpublished data). In vivo studies also revealed that CD45+/Col1+ cells might be involved in the progression of diabetic nephropathy through CCL2/CCR2 signaling (unpublished data).

In conclusion, CD45+/Col1+ cells, emerging effector cells derived from bone marrow, may be involved in the progression of diabetic nephropathy through the interaction with macrophages and CCL2/CCR2 signaling. Further studies for biology of bone-marrow-derived cells and the interaction of resident cells would be required for better understanding and the therapeutic strategies for diabetic nephropathy.

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Conflict of interest: **The authors declare no conflicts of interest.**

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Original Article

Matrix metalloproteinase-2 (MMP-2) and membrane-type 1 MMP (MT1-MMP) affect the remodeling of glomerulosclerosis in diabetic OLETF rats

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Abstract

Background. We reported previously that diabetic glomerular nodular-like lesions were formed during the reconstruction process of mesangiolysis. However, the precise mechanism has yet to be elucidated. Here, we investigated the roles of matrix metalloproteinase (MMP)-2, which is activated from proMMP-2 by membrane-type (MT)-MMP in the sclerotic and endothelial cell injury process of a type II diabetic model, Otsuka Long–Evans Tokushima Fatty (OLETF) rats.

Methods. Monocrotaline (MCT) or saline only was injected three times every 4 weeks in 36-week-old OLETF rats and control Long–Evans Tokushima Otsuka rats. Glomerular expression and enzymatic activity of MMP-2 and MT1-MMP were assessed by immunohistochemistry, gelatin zymography of cultured glomerular supernatants, *in situ* enzymatic detection and reverse transcription–polymerase chain reaction.

Results. Mesangial matrix increased in OLETF rats. In addition, mesangiolysis and nodular-like mesangial expansion were observed only in MCT-injected endothelial injured OLETF rats. MMP-2 and MT1-MMP proteins increased in the expanded mesangial lesions in OLETF 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 MCT-injected OLETF rats.

Conclusion. These results suggest that MMP-2 and MT1-MMP are produced and activated in glomeruli through the progression of diabetic nephropathy and may have some effect on the remodeling of the glomerular matrix in diabetic nephropathy.

Keywords: diabetic nephropathy; glomerulosclerosis; matrix metalloproteinase-2; membrane-type 1 matrix metalloproteinase; diabetic nodular-like lesion

Introduction

Diabetic nephropathy is one of the most important causes of end-stage renal disease. Characteristic pathological changes of diabetic nephropathy include accumulation of extracellular matrix (ECM) in glomerular and tubulointerstitial tissues. These pathological abnormalities are speculated to be induced by alterations in ECM production or degradation. Fioretto *et al.* [1] reported that 10 years of normoglycemia after pancreas transplantation ameliorated the lesions of diabetic nephropathy, suggesting the importance of degradation of accumulated ECM in diabetic nephropathy.

The glomerular ECM, which composes the glomerular basement membrane and mesangial matrix, has highly specialized mechanical and biological functions in the renal glomeruli [2, 3]. These functions are maintained by tight regulation of the balance between the rates of synthesis and degradation of ECM. Alterations of this balance may lead to pathological changes in glomerular structure and function. Therefore, studies on the regulatory mechanisms of ECM are very important to understand the pathogenesis of glomerular injury associated with qualitative and quantitative changes of ECM [4].

The degradation of ECM, including type IV and V collagens, laminin, fibronectin and proteoglycans, is mediated by proteinases, such as aspartic, cysteine, serine proteinases and matrix metalloproteinases (MMPs) [5]. In fact, the glomerular expression of MMPs, including MMP-2, 3, 9, 13, 14, 24, 25, 27 and 28, and membrane-type MMP (MT1, 2 -MMP) has been reported in various glomerular diseases, including diabetic nephropathy [6–15]. Among these MMPs, MMP-2 has a specific activation mechanism. MMP-2 is activated on the cell membrane by MT-MMP [16]. Among these MMPs, MMP-2 has a specific activation

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mechanism. MMP-2 is activated on the cell membrane by MT-MMP.

Here, we investigated the glomerular expression of MMP-2, MT1-MMP and *in situ* activity of MMP-2 in diabetic nephropathy using a diabetic model, Otsuka Long–Evans Tokushima Fatty (OLETF) rats, which resembles human type 2 diabetes mellitus [17–19]. In addition, we also attempted to induce nodular-like lesions resembling those seen in human patients through vascular endothelial injury and mesangiolysis by administration of monocrotaline.

Materials and methods

Animals and experimental design

OLETF rats and Long–Evans Tokushima Otsuka (LETO) rats used as controls were donated from Tokushima Laboratory of Otsuka Pharmaceutical Co. Ltd. We administered monocrotaline (2% fluid; Sigma, St Louis, MO) subcutaneously at a dose of 30 mg/kg three times every 4 weeks to 36-week-old male OLETF and LETO rats. Control rats were injected with the same amounts of saline three times. The rats were sacrificed at 40, 44, 46 and 50 weeks old for histological examination of kidney tissues and isolation of glomeruli for culture and RNA purification. There were no pathological changes in the lung or liver of monocrotaline-injected control LETO rats. There were also no differences in body features between monocrotaline-injected or non-injected control LETO rats.

All procedures used in the animal experiments complied with the standards set out in the ‘Guideline for the Care and Use of Laboratory Animals in Takara-machi Campus of Kanazawa University’.

Measurement of urinary albumin excretion

At 36, 46 and 50 weeks old, we examined urinary albumin excretion using 24-h urine collection. Measurements were performed by enzyme-linked immunosorbent assay (Nephrat; Exocell, Inc., Philadelphia, PA).

Histological examinations

Renal tissues were fixed in 10% buffered formalin, followed by embedding in paraffin and staining with hematoxylin and eosin, periodic acid-Schiff (PAS) reagent and periodic acid silver methenamine (PAM). Two independent observers with no prior knowledge of the experimental design evaluated each section. To avoid variation in the shape of the glomeruli, we chose glomeruli of the same diameter from a vascular pole. The degree of glomerular matrix expansion was expressed as an index, graded on an arbitrary scale as follows: 0, minor glomerular abnormalities; 1, segmental expansion by PAS-positive materials; 2, global expansion by PAS-positive materials without capillary tuft occlusion; 3, global expansion by PAS-positive materials with capillary tuft occlusion and 4, global sclerosis or hyalinosis. The numbers of glomeruli scored as X were Nx, and glomerular matrix score was calculated as $(0 \times N0 + 1 \times N1 + 2 \times N2 + 3 \times N3 + 4 \times N4)/50 \times 100$ $(N0 + N1 + N2 + N3 + N4 = 50)$. The numbers of nodular and mesangiolytic lesions were evaluated by two independent observers with no prior knowledge of the experimental design. In addition, a portion of each renal specimen was fixed with glutaraldehyde and osmium tetroxide, embedded in Epon (Epok) 812 (Oken Shoji Co., Tokyo, Japan), cut into 0.1-µm sections, double stained with uranyl acetate and lead citrate and examined with a Hitachi H-600 electron microscope (Hitachi Co, Tokyo, Japan).

Immunohistochemical detection of MMP-2 and MT1-MMP

Fresh specimens were embedded in optimal cutting temperature compound, snap frozen in *n*-hexane cooled with a mixture of dry ice and acetone and cut into 6-µm sections on a cryostat (Tissue-Tek II systems; Miles, Naperville, IL). Anti-human MMP-2 (clone 42-5D11) and anti-human MT1-MMP (clone 113-5B7) mouse monoclonal antibodies (Fuji Pharma Co., Ltd., Takaoka, Japan) were used as the primary antibodies. The cross-reactivity of these antibodies to rat molecules was confirmed by western blot analysis (data not shown). Immunohistochemistry was performed by the indirect avidin–biotin alkaline phosphatase method (Vectastain ABC-AP kit; Vector Labs, Burlingame, CA). Normal mouse IgG was used as a negative control. Human synovial tissues resected from patients with rheumatoid arthritis were used as positive controls for MMP-2 and MT1-MMP staining. The percentage MMP-2- or MT1-MMP-positive area in each glomerulus was evaluated using NIH Image.

Messenger RNA (mRNA) was extracted from formalin-fixed paraffin-embedded tissue using an Isogen PB Kit (Nippon Gene, Tokyo, Japan). MMP-2 mRNA expression was evaluated by real-time polymerase chain reaction (PCR) and normalized relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Glomerular isolation and culture

Rats were anesthetized with diethyl ether and the kidneys were perfused with saline *via* the aorta and then excised. The capsules were removed and the cortex was separated from the medulla and minced with a razor blade. Glomeruli were isolated by the differential sieving technique. The tissue was passed sequentially through 250-, 150- and 106-µm sieves. Intact glomeruli retained on the 106-µm sieve were washed with saline and resuspended in serum-free RPMI 1640 medium (Gibco, New York, NY) containing 100 U/mL of penicillin (Gibco), 100 U/mL of streptomycin (Gibco) and 0.2% lactalbumin (Sigma) in 12-well multi-well plates. The purity of the final suspension was determined by light microscopy. On average, there were fewer than five tubular fragments per 100 glomeruli. After 24 h of incubation at 37°C in 5% CO_2 in air, the culture media were removed.

In vitro and in vivo detection of MMP activity by zymography

The *in vitro* activities of MMPs in glomerular culture medium from LETO and OLETF rats was detected by zymography. Briefly, media were diluted appropriately to normalize for glomerular number. Gel loading buffer [0.5 M Tris–HCl, pH 6.8, 10% sodium dodecyl sulfate (SDS), 50% glycerol and 0.1% bromophenol blue] was added to the culture supernatant, and electrophoresis was performed on SDS–polyacrylamide gels (8%) containing 0.8 mg/mL gelatin (Sigma) under non-reducing conditions [20]. After electrophoresis, the gels were incubated in 2.5%Triton X-100 solution for 1 h at room temperature and then in a buffer containing 50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 10 mM $CaCl_2$ and 0.02% NaN_3 for 24 h at 37°C. The gels were stained with Coomassie brilliant blue, destained and photographed [21]. The zones of lysis were visualized and analyzed by densitometry. SDS–polyacrylamide gel electrophoresis (PAGE) molecular weight standards low and high (Bio-Rad, Richmond, VA) were used as molecular weight markers.

In situ detection of MMP activity on glomeruli was examined as follows. Renal frozen specimens were incubated with 1 mM synthetic peptides NFF-2 (MOCAc-Arg-Pro-Lys-Pro-Tyr-Ala-Nva-Trp-Met-Lys(Dnp)-NH_2; Peptide Institute, Osaka, Japan) resolved in dimethylsulfoxide (Sigma) and incubation buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10 mM $CaCl_2$, 0.02% NaN_3 and 0.05% Brij35) with 2 mM aminophenyl mercuric acetate as a substrate of MMP-2 for 48 h at 37°C and examined them with an immunofluorescence microscope using a U-MWU filter (AX-80; Olympus, Tokyo, Japan) [22].

Detection of MMP-2, MT1-MMP, TIMP-2 and fibronectin transcripts in isolated glomeruli

We performed semiquantitative analysis of the glomerular MMP-2, MT1-MMP, TIMP-2 and fibronectin gene expression by reverse transcription–polymerase chain reaction (RT–PCR). Total RNA was purified from isolated glomeruli by acid guanidinium isothiocyanate–phenol–chloroform extraction [23]. Sense and antisense primers, designed using the previously published complementary DNA sequence for rat MMP-2, rat GAPDH, rat MT1-MMP, TIMP-2 and fibronectin, were synthesized. Primers for MMP-2, MT1-MMP and TIMP-2 were designed using the primer program GENETYX-MAC (GENETYX, Tokyo, Japan). Primer pairs were chosen to yield an expected product of 248 bp for rat MMP-2, 363 bp for rat GAPDH [24], 398 bp for rat MT1-MMP, 603 bp for rat TIMP-2 and 294 bp for rat fibronectin [25] (Table 1). Aliquots of 1 µg of total RNA were subjected to reverse transcription. PCR was then performed using an RNA PCR kit (AMV; Takara, Kyoto, Japan). The thermal cycler was programmed with an initial incubation of 94°C for 1 min, followed by 35 – 40 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. The entire reaction mixture was subjected to 2% agarose gel electrophoresis, and PCR products were visualized with ethidium bromide staining and ultraviolet transillumination and were analyzed semiquantitatively by densitometry. RT–PCR data were normalized relative to the housekeeping gene *GAPDH* as a control.

Statistical analysis

Data are presented as means ± SEMs. Statistical significance of differences was assessed by analysis of variance (ANOVA) and the Kruskal–Wallis test. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

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Results

Urinary albumin excretion was increased in diabetic OLETF rats but was not influenced by monocrotaline

The urinary albumin excretion in control LETO rats was 1.5 ± 0.5, 1.2 ± 0.1 and 1.0 ± 0.3 mg/day at 36, 46 and 50 weeks old, respectively. However, the urinary albumin excretion in diabetic OLETF rats was significantly elevated as compared to that of LETO rats (86.6 ± 30.6, 155.0 ± 22.7, 112.0 ± 16.4 mg/day at 36, 46 and 50 weeks old, respectively; $P < 0.01$ by ANOVA). After monocrotaline injection, there were no significant changes in urinary albumin excretion in either LETO or OLETF rats (Figure 1).

Monocrotaline-accelerated diabetic glomerulopathy in diabetic OLETF rats

There was no significant glomerular changes in LETO rats (13.3 ± 3.3 at 40 weeks, 2.9 ± 1.8 at 50 weeks) (Figure 2). After monocrotaline injection, mild mesangial matrix expansion was seen in LETO rats (27.5 ± 11.1 at 40 weeks, 8.6 ± 4.6 at 50 weeks) (Figure 2). In contrast to LETO rats, marked mesangial expansion was observed in OLETF rats (166.7 ± 23.3 at 40 weeks, 154.0 ± 28.4 at 50 weeks) (Figure 2). Moreover, monocrotaline treatment in OLETF rats significantly increased glomerular matrix scores at 50 weeks (140.0 ± 17.3 at 40 weeks, 160.0 ± 4.1 at 44 weeks, 205.0 ± 18.5 at 46 weeks, 219.3 ± 6.0 at 50 weeks) (Figure 4A). In addition, some glomeruli showed typical mesangiolytic lesions and nodular-like lesions (Figure 2) in monocrotaline-treated OLETF rats at 50 weeks, concomitant with glomerular capillary endothelial cell swelling (Figure 3). Similar to mesangial matrix expansion, quantitative analysis indicated that nodular-like lesions and mesangiolysis gradually increased in monocrotaline-treated OLETF rats (Figure 4B and C).

MMP-2 and MT1-MMP proteins were upregulated in monocrotaline-treated diabetic OLETF rats

MMP-2 and MT1-MMP proteins were only faintly detected on the mesangial area of the glomeruli of LETO and monocrotaline-treated LETO rats by immunohistochemical study (data not shown). In OLETF rats, both proteins were observed mainly in the mesangial area and were enhanced in the area of severely expanded mesangial matrix. In monocrotaline-treated OLETF rats, the expression levels of both proteins were upregulated in the expanded mesangial areas and the segmental sclerotic lesions of glomeruli (Figure 5A and B).

Monocrotaline-treated diabetic OLETF rats showed in vivo activity of MMP-2 in glomeruli

We examined the gelatinolytic activity of glomerular culture media from 50-week-old LETO and OLETF rats by zymography using gelatin-substrate SDS–PAGE (Figure 6A and B). In the culture media from 50-week-old LETO rats, there were gelatinolytic activities at 62 and 68 kDa. The 62-kDa band was thought to indicate the activated form of MMP-2. The activities of 62 and 68 kDa were enhanced in monocrotaline-treated LETO (2.3- and 2.3-fold, respectively), saline-injected OLETF (4.8- and 2.7-fold, respectively) and monocrotaline-treated OLETF rats (5.5- and 3.1-fold, respectively). In addition, *in situ* MMP activity was detected in the mesangial areas of the glomeruli from the monocrotaline-treated OLETF rats at 50 weeks (Figure 6C) but not in the other groups.

Transcription of MMP-2 mRNA was enhanced more than those of TIMP-2 and fibronectin by monocrotaline treatment, especially in diabetic OLETF rats

MMP-2, MT1-MMP, TIMP-2 and fibronectin mRNAs were detected in isolated glomeruli by RT–PCR (Figure 7A). Optical densities of MMP-2, MT1-MMP, TIMP-2 and fibronectin RT–PCR products were corrected relative to those of GAPDH as an internal control in the same lane. Compared with the saline-injected LETO rats, *MMP-2* gene expression was enhanced in monocrotaline-treated LETO, saline-injected OLETF and monocrotaline-treated OLETF rats by 15.8-, 2.8- and 18.1-fold, respectively. MT1-MMP mRNA was also increased by 1.9-, 1.1- and 2.8-fold in these rats, respectively. The levels of TIMP-2 and fibronectin mRNA were also increased by 1.4- and 2.2-fold, respectively, in monocrotaline-treated LETO rats, by 1.1- and 2.2-fold, respectively, in saline-injected OLETF rats and by 2.2- and 3.0-fold, respectively, in monocrotaline-treated OLETF rats in comparison with control saline-injected LETO rats (Figure 7B).

Table 1. Primer sequences and expected PCR product sizes

Rarget	Sequence	Product sizes
MMP-2	Sense 5′-GTCTTCCCCTTCACTTTTCTG-3 Antisense 5′-CGGAAGTTCTTGGTGTAGGTG-3	248 bp
MT1-MMP	Sense 5′-GACTGAGATCAAGGCCAATG-3′ Antisense 5′-TGTCATTCCCATTTAGATCC-3′	398 bp
TIMP-2	Sense 5′-CCAAAGCAGTGAGCGAGAAGGA-3′ Antisense 5′-CAGGAAGGGATGTCAAAGCTGG-3′	603 bp
Fibronectin	Sense 5′-TTATGACGACGGGAAGACCT-3′ Antisense 5′-GCTGGATGGAAAGATTACTC-3′	294 bp
GAPDH	Sense 5′-CAGAACATCATCCCTGCATCC-3′ Antisense 5′-CACCCTGTTGCTGTAGCCATA-3′	363 bp

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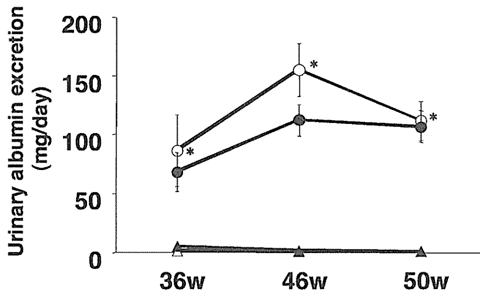

Fig. 1. Urinary albumin excretion increased in MCT-injected OLETF rats. Results in saline-injected LETO rats (open triangles) (36 weeks $n = 7$, 46 and 50 weeks $n = 4$), MCT-injected LETO rats (closed triangles) (36 weeks $n = 16$, 46 weeks $n = 10$, 50 weeks $n = 3$), saline-injected OLETF rats (open circles) (36 weeks $n = 7$, 46 and 50 weeks $n = 4$) and MCT-injected OLETF rats (closed circles) (36 weeks $n = 14$, 46 weeks $n = 7$, 50 weeks $n = 3$) are shown. Urinary albumin excretion significantly increased in saline-injected OLETF rats compared with saline-injected LETO rats but did not change with injection of MCT. *$P < 0.01$ versus saline-injected LETO rats by one-factor ANOVA.

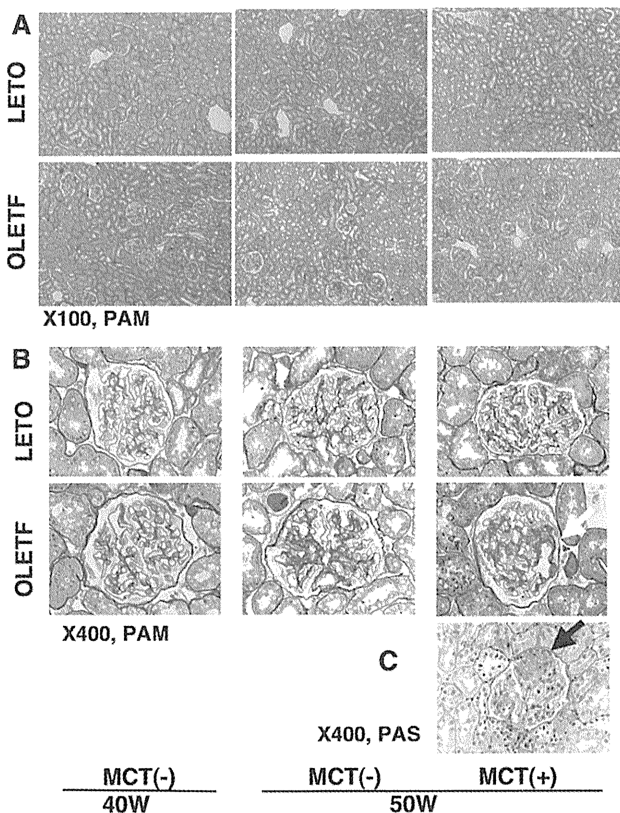

Fig. 2. Mesangiolysis and diffuse glomerulosclerosis were detected in an MCT-injected OLETF rat. Representative renal tissues obtained from a saline-injected LETO and OLETF rat (40 and 50 weeks), and MCT-injected LETO and OLETF rat (50 weeks) were demonstrated. Mesangial expansion was not seen in LETO rats at 50 weeks. The increase in mesangial expansion was evident in saline-injected OLETF rats at 50 weeks. Mesangiolysis (white arrow) and diffuse glomerulosclerosis associated with nodular-like mesangial expansion (black arrow) resembling human diabetic nodular-like lesions were seen in an MCT-injected OLETF rat at 50 weeks. (**A**) PAM stain, ×100; (**B**) PAM stain, ×400; (**C**) PAS stain, ×400.

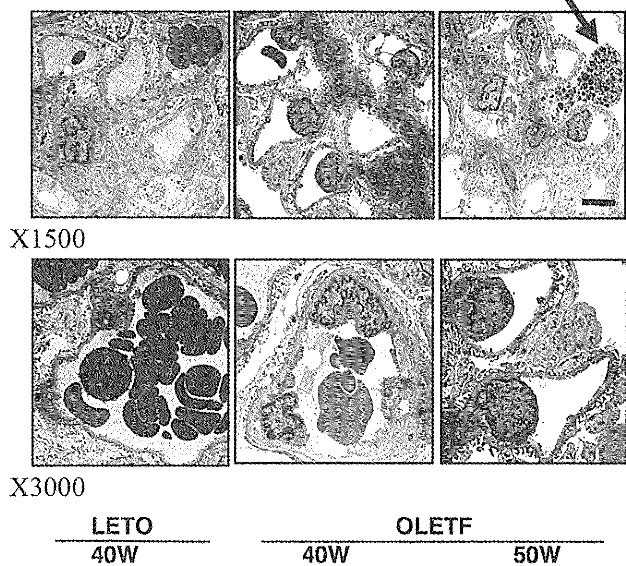

Fig. 3. Representative electron micrograph of the glomeruli. Electron micrographs of the glomeruli from MCT-injected LETO or OLETF rats at 40 and 50 weeks. Electron microscopic examination of MCT-injected OLETF rats at the age of 50 weeks revealed glomerular endothelial cell swelling and marked protein droplets in epithelial cells (arrow). Scale bar indicates 0.5 μm.

Discussion

In this study, we produced nodular-like lesions of diabetic glomerulopathy in a diabetic model with administration of monocrotaline. Diabetic OLETF rats, which present symptoms similar to those of human type 2 diabetes mellitus, showed vascular endothelial injury and mesangiolysis following administration of monocrotaline. Our results also indicated glomerular expression of MMP-2, MT1-MMP and *in situ* activity of MMP-2 in the diabetic nephropathy model. These data suggested that glomerular MMP-2 and MT1-MMP may play some role in the process of glomerular matrix remodeling in the progression of diabetic nephropathy.

The renal histology of OLETF rats mimics that of human diabetic nephropathy [17–19]. However, typical nodules in human diabetic nephropathy, which have a laminar structure and show weak positive PAM staining, mainly composed of type VI collagen were faintly seen in the kidneys of this rat model. Previously, we hypothesized that these human diabetic nodules were formed during the reconstruction of mesangiolytic lesions [26]. In this study, we attempted to produce human diabetic nodule-like lesions by administration of monocrotaline, which can induce mesangiolysis by glomerular capillary endothelial injury. Although the lamination of nodules in this model was incomplete, the nodule-like lesions resembling human diabetic nodules were seen in some glomeruli of monocrotaline-treated OLETF rats as well as diffuse glomerulosclerosis. An experimental model for nodular-like lesions was reported previously by Inagi *et al.* [27] utilizing megsin overexpression in RAGE and inducible nitric oxide synthase transgenic mice. However, these nodule-like lesions in monocrotaline-treated OLETF rats may be formed due to the influence of hyperglycemia

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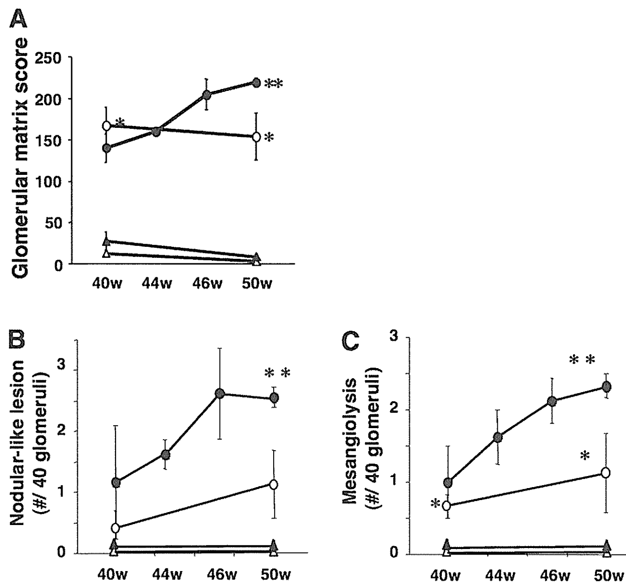

Fig. 4. Injection of MCT markedly increased the glomerular matrix score, nodular-like lesion and mesangiolysis in OLETF rats. Expanded extracellular matrix in mesangial area was semiquantitatively determined in each group of rats as glomerular matrix score. The results for saline-injected LETO rats (open triangles) (40 weeks $n = 3$ and 50 weeks $n = 4$), MCT-injected LETO rats (closed triangles) (40 weeks $n = 4$ and 50 weeks $n = 3$), saline-injected OLETF rats (open circles) (40 weeks $n = 3$ and 50 weeks $n = 4$) and MCT-injected OLETF rats (closed circles) (40 weeks $n = 3$, 44 weeks $n = 4$, 46 weeks $n = 4$ and 50 weeks $n = 3$) are shown. Glomerular matrix score was significantly increased in saline-injected OLETF rats compared with saline-injected LETO rats. The injection of MCT after the age of 36 weeks markedly increased the glomerular matrix score in OLETF rats compared with saline-injected OLETF rats at the age of 50 weeks (**A**). Similarly, the injection of MCT after the age of 36 weeks markedly increased the nodular-like lesions (**B**) and mesangiolysis (**C**) in OLETF rats compared with saline-injected OLETF rats at the age of 50 weeks. *$P < 0.01$ versus saline-injected LETO rats; **$P < 0.01$ versus saline-injected OLETF rats by Kruskal–Wallis rank test.

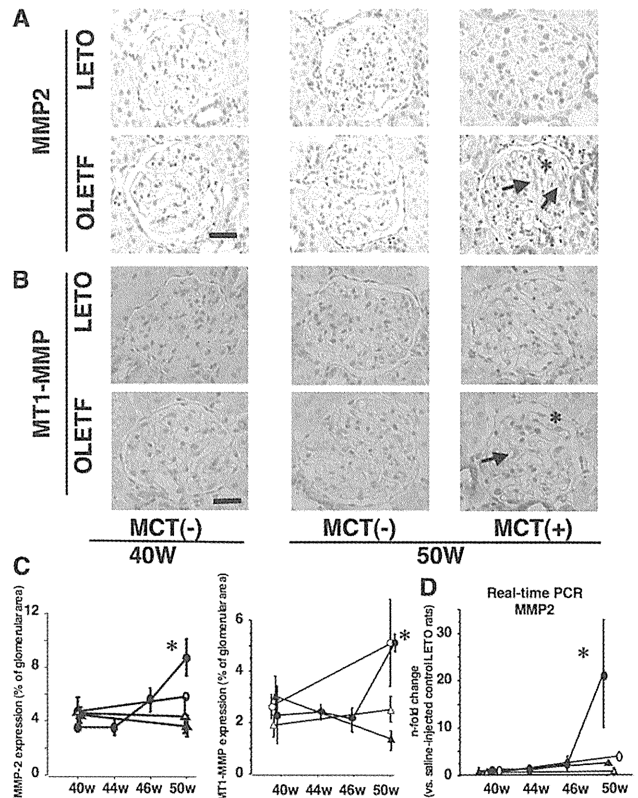

Fig. 5. MMP-2 and MT1-MMP protein in an MCT-injected OLETF rats were observed in the expanded mesangial areas and the glomerular visceral epithelial cells. Immunohistochemical analyses were performed as described in Methods using anti-MMP-2 (**A**) and anti-MT1-MMP (**B**) murine monoclonal antibodies. Renal tissues obtained from saline-injected (40 and 50 weeks) or MCT-injected LETO and OLETF rats (50 weeks) were evaluated. MMP-2 and MT1-MMP protein were observed in the mesangial cells of the expanded mesangial areas (arrows) and the glomerular visceral epithelial cells (asterisks). MMP-2 and MT1-MMP-positive area in glomerular area were quantitatively determined in each group of rats (**C**). MMP-2 mRNA expression was evaluated by real-time PCR. Results for saline-injected LETO rats (open triangles) (40 weeks $n = 3$ and 50 weeks $n = 4$), MCT-injected LETO rats (closed triangles) (40 weeks $n = 4$ and 50 weeks $n = 3$), saline-injected OLETF rats (open circles) (40 weeks $n = 3$ and 50 weeks $n = 4$) and MCT-injected OLETF rats (closed circles) (40 weeks $n = 3$, 44 weeks $n = 4$, 46 weeks $n = 4$ and 50 weeks $n = 3$) are shown. *$P < 0.05$ versus saline-injected OLETF rats by Kruskal–Wallis rank test. Scale bar indicates 20 μm.

or metabolic factors during the process of reconstruction from mesangiolysis because monocrotaline-induced glomerulopathy in Sprague–Dawley rats presents with mesangiolysis, following repair or remodeling process, but not nodular-like lesions. Therefore, both diabetic conditions and some toxic events associated with monocrotaline injection would be necessary for the formation of nodular-like lesions in this rat model.

MMP-2 is the principal MMP involved in ECM degradation in the glomeruli. MMP activity is regulated through the following three steps: (i) transcription, (ii) activation of the latent form and (iii) inhibition by TIMPs. The first step is MMP-2 transcription. In the present study, we demonstrated that MMP-2 was mainly localized to the expanded mesangial area and some proportion of visceral epithelial cells in monocrotaline-treated OLETF rats. In addition, isolated glomeruli clearly showed that *MMP-2* gene expression, protein production and activation were locally upregulated. Previous *in vitro* studies indicated that visceral epithelial cells and glomerular endothelial cells could produce MMP-2 as well as mesangial cells [28, 29]. Moreover, mesangial cells were reported to be positive for MMP-2 in Schoenlein–Henoch nephritis and IgA nephropathy [30]. *MMP-2* gene was regulated by cell-specific transcriptional systems [31–33]. *MMP-2* gene expression in mesangial cells was speculated to be regulated by activation of extracellular signal-regulated kinases 1 and 2, plasminogen activator inhibitor -1, transforming growth factor (TGF)-β and renin/prorenin [34, 35]. Among these regulatory systems, TGF-β is well known as an inducer of MMP-2 in mesangial cells *in vitro* [11]. Moreover, TGF-β was suggested to play a central role in animal models of diabetic nephropathy as well as human diabetic nephropathy [36–39]. Furthermore, it has been reported that glomerular TGF-β and type IV collagen expression were increased in the kidneys of OLETF rats at 30 weeks old [40, 41]. In addition, MMP-2 mRNA was upregulated coincident with the enhancement of TGF-β mRNA in anti-Thy1.1 glomerulonephritis model [11]. These data indicate that MMP-2 may have some role in the pathogenesis of diabetic nephropathy under conditions of TGF-β

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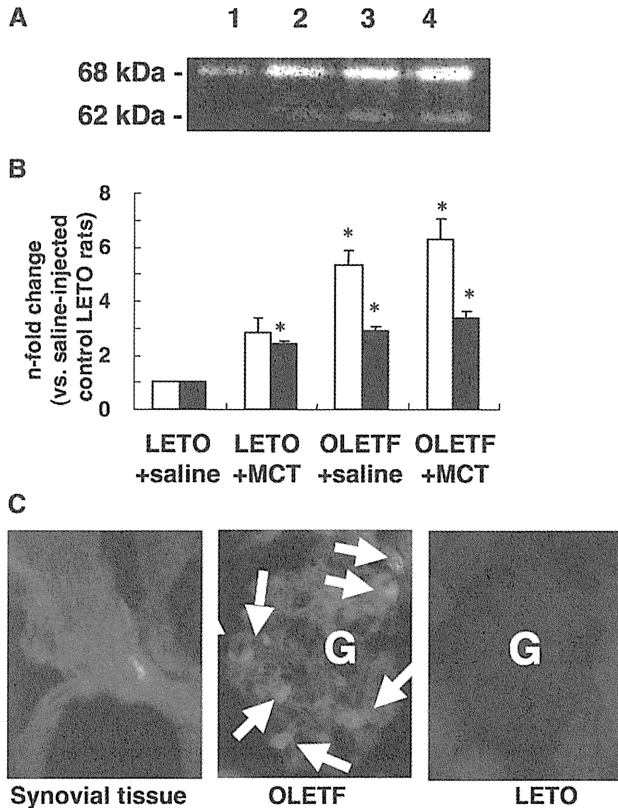

Fig. 6. Gelatinolytic activity of MMP-2 was increased in MCT-injected OLETF rats. Conditioned media obtained from isolated glomeruli from each group of rats at the age of 50 weeks cultured for 24 h in serum-free RPMI 1640 were subjected to gelatin zymography. (**A**) Polyacrylamide gel containing gelatin shown is representative of electrophoresis followed by Coomassie brilliant blue staining. Lane 1, conditioned media from saline-injected LETO rats; Lane 2, MCT-injected LETO rats; Lane 3, saline-injected OLETF rats and Lane 4, MCT-injected OLETF rats. (**B**) Analysis of gelatinolytic activity of MMP-2. Values are expressed as *n*-fold increase versus saline-injected LETO rats. open bars, 62 kDa; closed bars, 68 kDa. *P < 0.05 versus saline-injected LETO rats. (**C**) *In situ* detection of MMP activities was performed on renal tissues obtained from MCT-injected OLETF rats at the age of 50 weeks. Synovial tissue was used as a positive control and glomeruli of LETO rats were used as negative controls. Blue fluorescent deposits (arrows) resulting from cleavage of MOCAc-Arg-Pro-Lys-Pro-Tyr-Ala-Nva-Trp-Met-Lys (Dnp)-NH_2 were observed in the mesangial areas; G, glomerulus. Scale bar indicates 20 μm.

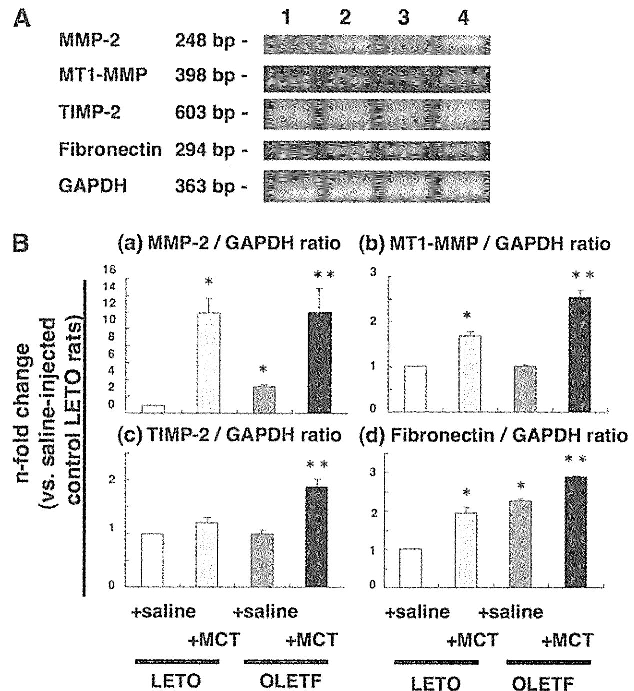

Fig. 7. Levels of MMP-2, MT1-MMP, TIMP-2 and fibronectin2 gene expression were increased in MCT-injected OLETF rats. Total RNA was extracted from isolated glomeruli in each group of rats at the age of 50 weeks. (**A**) Agarose gels shown are representative of electrophoresis followed by ethidium bromide staining. Lane 1, saline-injected LETO rats; Lane 2, MCT-injected LETO rats; Lane 3, saline-injected OLETF rats and Lane 4, MCT-injected OLETF rats. Expected sizes of RT–PCR products were 248 bp for rat MMP-2, 398 bp for rat MT1-MMP, 603 bp for rat TIMP-2, 294 bp for rat fibronectin and 363 bp for rat GAPDH. (**B**) Semi-quantification of MMP-2, MT1-MMP, TIMP-2 and fibronectin mRNA levels. Results for saline-injected ($n = 4$) or MCT-injected ($n = 3$) LETO rats and saline-injected ($n = 4$) or MCT-injected ($n = 3$) OLETF rats are shown. The bands were normalized relative to GAPDH expression. Values are expressed as *n*-fold increase versus saline-injected control LETO rats. (a) MMP-2; (b) MT1-MMP; (c) TIMP-2 and (d) fibronectin. *P < 0.05 versus saline-injected LETO rats, **P < 0.05 versus saline-injected OLETF rats.

stimulation. In future, blocking studies of TGF-β may be helpful to confirm the *in vivo* inducers of MMP-2 in this OLETF rat model.

The second regulatory step of MMP is activation. We detected MT1-MMP, which is a proMMP-2 activator, in the expanded mesangial area and glomerular visceral epithelial cells, and the distribution of this protein was coincident with that of MMP-2. Cultured mesangial cell can produce MT1-MMP [42]. Moreover, MT1-MMP mRNA was detected in the mesangial area of anti-Thy1.1 glomerulonephritis, using *in situ* hybridization [21]. Although McLennan *et al.* [43] reported that cultured mesangial cell-derived *MT1-MMP* gene expression was reduced by high glucose concentration, the glomerular expression levels of this gene in OLETF and LETO rats were not different in our study. In addition, glomerular MT1-MMP mRNA levels of both OLETF and LETO rats were increased by monocrotaline injection, coinciding with the increase of activated MMP-2 protein. Thus, MT1-MMP may play a role in glomerular MMP-2 activation. In addition, MT1-MMP itself can degrade type I and III collagens which accumulates in sclerotic glomeruli [44]. These observations suggest that MT1-MMP may play a dual role in digestion of ECM through direct cleavage of the substrates and the activation of proMMP-2 in diabetic glomeruli.

The third regulatory step of MMP involves inhibition by TIMPs. Among the TIMP family, TIMP-2 plays a major role in inhibiting MMP-2 activity. We demonstrated that both MMP-2 and TIMP-*2* gene and protein expression levels were increased in monocrotaline-treated OLETF rats, and the upregulation of MMP-2 mRNA expression was much higher than those of TIMP-2. We also detected MMP-2 enzyme activation in glomeruli of monocrotaline-treated OLETF rats. It was reported that TIMP-2 shows different roles for MMP-2 activation depending on its concentration; high concentrations of TIMP-2 inhibit proMMP-2

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and MMP-2 activation, while low concentrations activate proMMP-2 concomitant with MT-MMP [45]. Moreover, *TIMP-2* and *MMP-2* gene expression in mesangial cells were regulated by glucose concentration *in vivo* and *in vitro* [9, 43, 46]. Under these complicated control systems, MMP-2 activity overcame TIMP inhibition, and MMP-2 enzyme activity may play some role in ECM degradation associated with diabetic nephropathy.

Finally, the balance between ECM production and degradation is important for progression of diabetic nephropathy. Although the MMP-2 activity was enhanced in the glomeruli, glomerulosclerosis (ECM accumulation) progressed in monocrotaline-treated OLETF rats. These results suggested that ECM production may overcome ECM degradation in monocrotaline-treated OLETF rats. It has been reported that type III, IV and VI collagen, fibronectin and laminin expression are increased at the transcript and protein levels in the presence of high glucose concentrations [19, 47]. Moreover, hemodynamic abnormalities such as glomerular hypertension and hyperglycemia-induced metabolic disorders, such as advanced glycation end product production, polyol pathway abnormalities and protein kinase C activation, will stimulate ECM production. Furthermore, normalization of hyperglycemia-ameliorated glomerular diffuse lesions, suggesting that the degradation system of accumulated ECM was dominant under normoglycemic conditions [1]. These observations suggest that overproduction of ECM induced by hyperglycemia may play an important role in the progression of diabetic nephropathy.

In summary, MMP-2 and MT1-MMP are produced and activated in glomeruli through the progression of diabetic glomerulosclerosis and may play important roles in the remodeling process of glomerular matrix in the diabetic nephropathy model of monocrotaline-treated OLETF rats. The altered synthesis of MMP, TIMP and ECM components will determine the outcome of ECM metabolism.

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Conflict of interest statement. None declared.

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