Experimental diabetic nephropathy is accelerated in matrix metalloproteinase-2 knockout mice

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Abstract

Background. Matrix metalloproteinase-2 (MMP-2) is responsible for the degradation of various types of extracellular matrix (ECM) proteins such as type IV collagen. Decreased MMP-2 expression and activity has been generally thought to contribute to increased accumulation of ECM at the advanced stage of diabetic nephropathy. However, the kinetics and role of MMP-2 in the early phase of diabetic nephropathy remain unclear. To address this issue, we examined whether streptozotocin (STZ)-induced early diabetic nephropathy was accelerated in MMP-2 knockout (KO) mice.

Methods. Diabetes was induced by the injection of STZ in 6-week-old control and MMP-2 KO mice. Animals were killed after 16 weeks of diabetes or after observation alone.

Results. Compared with non-diabetic control mice, renal MMP-2 expression and activity were increased in 16-week-old diabetic mice. Serum levels of blood urea nitrogen and creatinine and urinary excretion levels of albumin and N-acetyl-β-D-glucosaminidase were significantly elevated in diabetic MMP-2 KO mice when compared with wild-type diabetic littermates. Further, accumulation of ECM in the glomeruli and atrophy and fibrosis in the tubulointerstitial areas of diabetic MMP-2 KO mice were exacerbated, and renal α-smooth muscle actin expression was enhanced in diabetic MMP-2 KO mice.

Conclusions. Our present study suggests that renal expression and activity of MMP-2 are increased as a compensatory mechanism in the early phase of diabetic nephropathy. Since MMP-2 could play a protective role against the progression of diabetic nephropathy, further enhancement of MMP-2 expression and/or activity in the kidney may be a therapeutic target for the treatment of early diabetic nephropathy.

Keywords: albuminuria; diabetic nephropathy; extracellular matrix; MMP-2; tubulointerstitial injury
in early diabetic nephropathy, we assessed renal injury in a model of STZ-induced diabetic nephropathy in the context of genetic deletion of MMP-2.

Materials and methods

Animals

Mice deficient in MMP-2 [Ctrl-MMP-2 knockout (KO) mice, backcrossed onto a C57BL/6J] a gift from RIKEN BioResource Center [10], and bred in a specific pathogene-free facility (Kurume University School of Medicine, Kurume, Japan). Genetically normal C57BL/6J mice were used as an appropriate control. The studies were approved by the animal ethics committees of the Kurume University School of Medicine. Mice were randomized into non-diabetic and diabetic groups [C57BL/6J Ctrl (n = 13), Ctrl-MMP-2 KO (n = 9), C57BL/6J DM (n = 11) and DM-MMP-2 KO group (n = 9)]. Diabetes was induced by five daily intraperitoneal injections of STZ (50 mg/kg in citrate buffer, Sigma, St Louis, MO) in 6-week-old male mice. Only animals with blood glucose levels >300 mg/dL 7 days after diabetes induction were included in this study. Control mice received citrate buffer alone. Animals had unrestricted access to water and standard mouse chow, and were maintained on a 12-h light–dark cycle.

DNA genotyping was performed by the polymerase chain reaction (PCR) method for all the experimental mice. MMP-2 KO mice were genotyped using primers for the MMP-2 gene, forward primer: CAAC GATGGAGGCAGAGTA, reverse primer: GCCGGGGAACCTGGCATGG. After 16 weeks of diabetes, blood pressure (BP) was measured using a tail-cuff method (Model MK-2000 Storage Pressure Meter, Muramachi Kikai, Tokyo, Japan), and animals were anesthetized with an intraperitoneal injection of pentobarbital. Then the kidneys were dissected, frozen and stored at −80°C or in buffered formalin (4%). Blood was collected, centrifuged and plasma was obtained and stored at −80°C or in 6-week-old male mice. Only animals with blood glucose levels >300 mg/dL 7 days after diabetes induction were included in this study. Control mice received citrate buffer alone. Animals had unrestricted access to water and standard mouse chow, and were maintained on a 12-h light–dark cycle.

Statistical analysis

All values were presented as means ± standard error. One-way analysis of variance (ANOVA) followed by the Tukey test was performed for statistical comparisons; P < 0.05 was considered significant. All statistical analyses were performed with SPSS software (SPSS version 19). Since albuminuria was not normally distributed, non-parametric ANOVA, followed by a Steel-Dwass test, was performed for the analysis of albuminuria (SAS version 9.2).

Results

Characteristics of animals

Compared with non-diabetic Ctrl mice, plasma glucose, HbA1c, systolic BP and Ccr were significantly elevated, while body weight was significantly lower in 16-week old DM mice (Table 1). Furthermore, compared with DM mice, although plasma glucose level was not significantly affected and HbA1c value was modestly decreased in DM-MMP-2 KO mice, serum levels of BUN and Cr were significantly elevated, whereas Ccr was significantly reduced in these mice. HbA1c levels in non-diabetic Ctrl-MMP-2 KO mice were also significantly lower than in Ctrl mice.

Gelatin zymography

Proteins were extracted from the kidneys by a heat extraction method [13]. In brief, frozen kidney cortex was lysed in protein extraction buffer (50 mmol/L Tris–HCl pH 7.6, 0.2 mol/L NaCl, 5 mmol/L CaCl₂, 0.02% Brij-35). Then the lysates were centrifuged (30 min, 4°C), and pellets were re-suspended into 0.1 mol/L CaCl₂ and heated at 60°C for 5 min, then concentrated by centrifugation (Microcon YM-10, Millipore, Bedford, MA) and stored at −80°C. Protein concentration was measured by BCA protein assay (Thermo Scientific, IL). Pre- and active MMP-2 levels were evaluated with gelatin zymography as described previously [14]. Gelatinase bands were quantified by CS analyser version 2.0 (AFTO Co., Tokyo, Japan).

Immunostaining of type IV collagen

Specimens of kidney cortex were fixed with 4% paraformaldehyde, embedded in paraffin, sectioned at 4-μm intervals and mounted on glass slides. The sections were incubated in 0.3% hydrogen peroxide methanol for 30 min to block endogenous peroxidase activity, and incubated overnight at 4°C with rabbit polyclonal antibodies (Abs) raised against type IV collagen (ab65856; 1:1000) (abcam, Tokyo, Japan). Then the reactions were visualized with an avidin-biotin alkaline phosphatase kit (Vectas-tain ABC-AP; Vector Laboratories Inc., CA). Immunoreactivity in 10 different interstitial fields (×400) in each sample was measured by image analysis software (Optimas version 6.57; Media Cybernetics, Silver Spring, MD).

Western blotting

Twenty micrograms of protein extracted from kidney cortex were subjected to SDS–PAGE and western blotting with specific primary Abs raised against mouse anti-α-smooth muscle actin (α-SMA) (NB600-531; 1:4000) (Novus Biologicals, LLC., CO) or monoclonal anti-β-actin (A-5441; 1:1000) (Sigma-Aldrich, CO., MO) and horseradish peroxidase-conjugated secondary Abs (NA931; 1:2000) (GE Healthcare, UK Ltd). Detection was performed by enhanced chemiluminescence (Thermo Scientific Inc.).
MMP-2 gene and protein expression levels were increased in the kidney of DM mice

MMP-2 is secreted by renal cells in an inactive form, pro-MMP-2 (72 kDa) and then activated on the cell surface [15]. Thus, we first examined MMP-2 gene and protein expression levels in Ctrl and DM mice. As shown in Figure 1, compared with Ctrl mice, MMP-2 mRNA level and expression of pro- and active form of MMP-2 were significantly increased in the kidney of 16-week DM mice. Renal MMP-2 mRNA level in DM mice was increased (~15-fold) when compared with Ctrl mice (Figure 1A). Consistent with the gene expression findings, gelatin zymography revealed that the active form of MMP-2 (62 kDa) in the kidney of DM mice was also elevated (~3-fold) when compared with Ctrl mice (Figure 1B). As expected, neither MMP-2 gene nor protein was detected in MMP-2 KO mice (Figure 1A and B).

The activity of MMP-2 is negatively regulated by TIMP-2 [15]. Therefore, we next investigated TIMP-2 mRNA levels in the kidneys from each group. Although TIMP-2 mRNA levels in the kidney of DM mice were significantly increased up to 2-fold (Figure 1C), the ratio of MMP-2 to TIMP-2 mRNA levels in DM mice was still significantly higher (~6-fold) (Figure 1D). MMP-2 deficiency did not affect TIMP-2 mRNA levels in Ctrl or DM mice (Figure 1C).

Albuminuria and mesangial matrix expansion were exacerbated in DM-MMP-2 KO mice

To elucidate the pathophysiological role of MMP-2 in the early stage of diabetic nephropathy, we examined the effect of deletion of MMP-2 gene on albuminuria and glomerular ECM accumulation in STZ-DM mice. The induction of diabetes resulted in increased albuminuria (Figure 2) and enhanced accumulation of ECM proteins in the mesangial areas, as assessed by Masson’s trichrome (Figure 3) and type IV collagen staining (Figure 4B–E). Furthermore, compared with DM mice, albuminuria and mesangial matrix expansion were further increased in DM-MMP-2 KO mice (Figures 2–4B–E). There were no significant differences in albuminuria or mesangial matrix expansion between Ctrl and Ctrl-MMP-2 KO mice.

Tubulointerstitial atrophy and fibrosis were exacerbated in DM-MMP-2 KO mice

Diabetes was associated with increased tubular atrophy, evaluated by measurement of tubulointerstitial area (Figure 5A–D) and by interstitial fibrosis with Masson’s trichrome staining (Figure 5E–H), respectively. The deletion of MMP-2 further deteriorated the tubulointerstitial changes. Consistent with the increased tubulointerstitial injury, there was functional evidence of tubular injury with increased NAG in DM mice which was further increased in DM-MMP-2 KO mice (Figure 6). Since type IV collagen α1 chain mRNA levels are increased in the tubulointerstitial and glomerular area of early diabetic nephropathy [16], we examined the effects of MMP-2 deficiency on type IV collagen expression. Type IV collagen α1 chain mRNA and protein levels were modestly, but significantly increased in DM mice (Figure 4A–E). In DM-MMP-2 KO mice, type IV collagen expression levels were further enhanced. α-SMA is expressed in damaged tubular cells [17]. Thus, we examined whether the deletion of MMP-2 could affect α-SMA expression levels in the kidney of DM mice. As shown in Figure 7, compared with Ctrl mice, α-SMA expression was increased in the kidney of DM mice, which was further enhanced in DM-MMP-2 KO mice.

Discussion

Since there was evidence of a marked increase in MMP-2 expression and activity in the kidneys of diabetic mice, the renal phenotype of diabetic MMP-2 KO mice was examined. Surprisingly, serum levels of BUN and Cr and urinary excretion levels of albumin and NAG were significantly elevated and Ccr was decreased in the diabetic MMP-2 KO mice, and these functional changes were associated with increased renal structural injury. This
included enhanced accumulation of ECM in the glomeruli and atrophy and fibrosis in the tubulointerstitium with increased renal α-SMA expression. This increase in functional and structural manifestations of diabetic nephropathy in these MMP-2 KO mice occurred despite a modest improvement in overall glycemic control, as reflected by a lower HbA1c and a tendency toward a lower plasma glucose concentration. These observations suggest

Fig. 1. MMP-2 mRNA levels and activity and TIMP-2 mRNA levels in each animal group. (A) MMP-2 mRNA expression in the renal cortex was evaluated by real-time quantitative RT–PCR. Ctrl mice; n = 13, Ctrl-MMP-2 KO mice; n = 7, DM mice; n = 10, DM-MMP-2 KO mice; n = 8. (B) Pro- and active MMP-2 levels in the renal cortex were determined by gelatin zymography. Ctrl mice; n = 7, Ctrl-MMP-2 KO mice; n = 7, DM mice; n = 6, DM-MMP-2 KO mice; n = 7. (C) Renal TIMP-2 mRNA levels were evaluated by real-time quantitative RT–PCR. Ctrl mice; n = 13, Ctrl-MMP-2 KO mice; n = 7, DM mice; n = 10, DM-MMP-2 KO mice; n = 8. (D) The ratio of MMP-2 to TIMP-2 mRNA in Ctrl and DM mice. Ctrl mice; n = 13, DM mice; n = 10. *P < 0.05, **P < 0.01 versus appropriate control mice.
that renal expression and activity of MMP-2 are increased as a compensatory mechanism in the early phase of diabetic nephropathy and indeed that MMP-2 could play a protective role against the progression of diabetic nephropathy.

Several reports have shown that glomerulosclerosis and tubulointerstitial fibrosis in the advanced stage of diabetic nephropathy is associated with the down-regulation of MMP-2 gene expression and activity in the kidney [8, 9, 18]. Specifically, Inada et al. [18] have shown that glomerular MMP-2 expression is decreased, which is accompanied by increased expression of type IV collagen and glomerulosclerosis in 40-week old diabetic mice. Further, McLennan et al. [8] reported that tubulointerstitial type IV collagen is increased in association with decreased MMP-2 activity in 6-month STZ-induced diabetic rats. The findings for these current studies suggest a close link between decreased MMP-2 activity and increased renal ECM accumulation in diabetes. However, it would be noted that changes in MMP-2 may be time dependent. Therefore, to determine the potential direct role of increased MMP-2 activity in the early stage of diabetic kidney disease, it was necessary to explore the renal changes in an experimental model of diabetes with the deletion of MMP-2. Indeed, the present study demonstrates that MMP-2 is a central player in regulating the accumulation of the key renal basement membrane collagen, type IV collagen in diabetes. Diabetic MMP-2 KO mice had increased renal type IV collagen accumulation without an increase in type IV collagen gene expression, suggesting a crucial role for MMP-2 activity in this setting. Furthermore, tubulointerstitial atrophy, increased excretion of NAG and albumin and renal functional impairment were accelerated in DM-MMP-2 KO mice. Even at this earlier stage of diabetic nephropathy, ECM accumulation in the mesangial and tubulointerstitial areas is increased in DM mice, whose pathological changes were accelerated in DM-MMP-2 KO mice. These observations suggest that MMP-2 gene expression could be induced and its enzymatic activity is enhanced in the kidney during the early phase of diabetes, but it may not be sufficient to prevent the excess accumulation of ECM proteins in the glomeruli and tubulointerstitium. In the present study, although TIMP-2 gene expression in the

![Fig. 2. Albuminuria in each animal group. Ctrl mice; n = 13, Ctrl-MMP-2 KO mice; n = 9, DM mice; n = 11, DM-MMP-2 KO mice; n = 9, **P < 0.01 versus appropriate control mice. #P < 0.05 versus DM mice.](image)

![Fig. 3. Glomerular ECM accumulation in each animal group. Glomerular ECM accumulation was evaluated by the intensity of Masson’s trichrome staining in the glomeruli. Representative kidney sections of 16-week (A) Ctrl mice; n = 6, (B) Ctrl-MMP-2 KO mice; n = 8, (C) DM mice; n = 6 and (D) DM-MMP-2 KO mice; n = 6. Magnification, ×600. *P < 0.05 versus appropriate control mice. #P < 0.05 versus DM mice.](image)

![Fig. 4. Urinary NAG levels in each animal group. Ctrl mice; n = 13, Ctrl-MMP-2 KO mice; n = 9, DM mice; n = 11, DM-MMP-2 KO mice n = 9, **P < 0.01 versus appropriate control mice. ##P < 0.01 versus DM mice.](image)
Fig. 5. Tubulointerstitial atrophy and fibrosis in each animal group. Tubulointerstitial atrophy and fibrosis were evaluated by the area covered by the interstitium with PAS staining (A–D) and Masson’s trichrome staining (E–H), respectively. Representative kidney sections of (A) Ctrl mice; n = 6, (B) Ctrl-MMP-2 KO mice; n = 8, (C) DM mice; n = 6, (D) DM-MMP-2 KO mice; n = 6, (E) Ctrl mice; n = 6, (F) Ctrl-MMP-2 KO mice; n = 8, (G) DM mice; n = 6 and (H) DM-MMP-2 KO mice; n = 6. Magnification, ×400. **P < 0.01 versus appropriate control mice. #P < 0.05 versus DM mice.

Fig. 6. Type IV collagen expression in the kidney. (A) Type IV collagen (α1) mRNA expression in the renal cortex was evaluated by real-time quantitative RT–PCR. Ctrl mice; n = 13, Ctrl-MMP-2 KO mice; n = 7, DM mice; n = 10, DM-MMP-2 KO mice; n = 8. (B–E) Tubulointerstitial type IV collagen staining in each animal group. Representative kidney sections of (B) Ctrl mice; n = 6, (C) Ctrl-MMP-2 KO mice; n = 8, (D) DM mice; n = 6 and (E) DM-MMP-2 KO mice; n = 6. Magnification, ×400. *P < 0.05, **P < 0.01 versus appropriate control mice. #P < 0.05 versus DM mice.
kidney was modestly increased in DM mice, the ratio of MMP-2 to TIMP-2 was still higher than that in non-diabetic mice. It is unlikely that overexpression of TIMP-2 in the kidney has overcome the enhanced activity of MMP-2 to modulate ECM accumulation in the early phase of diabetic nephropathy.

One of the characteristic features of early diabetic nephropathy is albuminuria, which is thought to be multifactorial including involvement of a result of glomerular epithelial cell injury, but is likely to be involved in further mechanisms including tubular handling of albuminuria [19]. In this study, we found that albuminuria was exacerbated in DM-MMP-2 KO mice. Lutz et al. [20] have reported that inhibition of MMPs results in significantly higher proteinuria in chronic allograft nephropathy in rats. Therefore, MMP-2 might be protective against albuminuria by inhibiting ECM accumulation not only in the glomerulus but also the tubulointerstitium.

In the present study, plasma glucose had a tendency to decrease and HbA1c levels were reduced in DM-MMP-2 KO mice compared with DM mice (Table 1). Thus, it is unlikely that the MMP-2 gene KO could accelerate glomerular and tubulointerstitial injury in the kidney of DM mice as a result of effects on glycemic control. HbA1c levels were also significantly decreased in Ctrl-MMP-2 KO mice when compared with Ctrl mice, suggesting that MMP-2 may have effects on glucose tolerance. In accordance with the present observations, Van Hul and Lijnen [21] have recently shown that MMP-2 KO mice kept on a high-fat diet had lower body weight, fat mass and plasma glucose concentrations when compared with wild-type littermates. It remains to be determined what the role of MMP-2 on glucose metabolism is in this study, but it appears that these metabolic effects warrant further investigation.

In the present study, MMP-2 gene KO itself did not affect tubulointerstitial atrophy, fibrosis and type IV collagen accumulation in Ctrl mice (Figures 4 and 5). Furthermore, urinary NAG levels were significantly higher in Ctrl-MMP-2 KO mice than those in Ctrl mice (Figure 6). Therefore, although the biological significance of increased NAG in Ctrl-MMP-2 KO mice remained unclear, urinary NAG levels may not only reflect histopathological changes in the tubulointerstitium, but could indicate that MMP-2 is involved in the maintenance of tubular function in vivo.

In conclusion, the present study suggests that renal expression and activity of MMP-2 are increased as a compensatory mechanism in the early phase of diabetic nephropathy. Since MMP-2 could play a protective role against the progression of diabetic nephropathy, further enhancement of MMP-2 expression and/or activity in the kidney may be a therapeutic target for the treatment of early diabetic nephropathy. Further in vivo studies using MMP-2 activator may be helpful in the mouse model of diabetic nephropathy.

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Conflict of interest statement. None declared.

References

High phosphate feeding promotes mineral and bone abnormalities in mice with chronic kidney disease

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Abstract
Background. Chronic kidney disease-mineral bone disorder (CKD-MBD) is a systemic syndrome characterized by imbalances in mineral homeostasis, renal osteodystrophy (ROD) and ectopic calcification. The mechanisms underlying this syndrome in individuals with chronic kidney disease (CKD) are not yet clear.

Methods. We examined the effect of normal phosphate (NP) or high phosphate (HP) feeding in the setting of CKD on bone pathology, serum biochemistry and vascular calcification in calcification-prone dilute brown noragouti (DBA/2) mice.

Results. In both NP and HP-fed CKD mice, elevated serum parathyroid hormone and alkaline phosphatase (ALP) levels were observed, but serum phosphorus levels were equivalent compared with sham controls. CKD mice on NP diet showed trabecular alterations in the long bone consistent with high-turnover ROD, including increased trabecular number with abundant osteoblasts and osteoclasts. Despite trabecular bone and serum biochemical changes, CKD/NP mice did not develop vascular calcification. In contrast, CKD/HP mice developed arterial medial calcification (AMC), more severe trabecular bone alterations and cortical bone abnormalities that included decreased cortical thickness and density, and increased cortical porosity. Cortical bone porosity and trabecular number strongly correlated with the degree of aortic calcification.

Conclusions. HP feeding was required to induce the full spectrum of CKD-MBD symptoms in CKD mice.

Keywords: chronic kidney disease-mineral bone disorder; high-turnover renal osteodystrophy; phosphate; vascular calcification

Introduction
The term chronic kidney disease-mineral bone disorder (CKD-MBD) is a term coined by the Kidney Disease Improving Global Outcomes Foundation [1] to describe the complex syndrome of mineral dysmetabolism, renal osteodystrophy (ROD) and extra-skeletal calcification that is unique to CKD. Recognition of this phenomenon is important due to the association between disturbances in mineral and bone metabolism and the increased risk of...