Cardiovascular, Pulmonary and Renal Pathology

Growth Factor Midkine Is Involved in the Pathogenesis of Diabetic Nephropathy

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Diabetic nephropathy is a life-threatening disease associated with diabetes mellitus. Longstanding hyperglycemia induces pathological reactions of glomerular mesangial cells, such as overproduction of extracellular matrix, which finally lead to nephropathy. However, the mechanisms underlying its pathogenesis have not been completely elucidated. Using the Streptozotocin-induced model of diabetes, we report that mice deficient in the growth factor midkine (*Mdk*^{-/-}) exhibited strikingly milder nephropathy than *Mdk*^{+/+} mice, even though both mice showed similar extents of hyperglycemia after Streptozotocin injection. Midkine expression was induced in the glomerular mesangium of *Mdk*^{+/+} mice with diabetic nephropathy and in primary cultured mesangial cells exposed to high glucose. *Mdk*^{-/-} mesangial cells exhibited reduced phosphorylation of protein kinase C and extracellular signal-regulated kinase as well as reduced production of transforming growth factor- β_1 on high glucose loading. Addition of exogenous midkine restored extracellular signal-regulated kinase phosphorylation in *Mdk*^{-/-} cells under high glucose conditions, whereas a midkine antisense oligodeoxynucleotide suppressed midkine in *Mdk*^{+/+} cells. Therefore, this study identifies midkine as a key molecule in diabetic nephropathy and suggests that midkine accelerates the intracellular signaling network evoked by hyperglycemia in nephropathy. (*Am J Pathol* 2006, 168:9–19; DOI: 10.2353/ajpath.2006.050488)

Diabetic nephropathy is a major complication of diabetes mellitus, which is the most common cause of end-stage

renal failure in many countries.^{1,2} Therefore, it is very important for the development of therapeutic strategies to elucidate the molecular mechanism underlying this disease. There is an increasing body of evidence that the pathogenesis involves many molecules, such as protein kinase C (PKC), extracellular signal-regulated kinase (ERK), transforming growth factor (TGF)- β_1 , and angiotensin II.^{3–5} The pathological characteristics of diabetic nephropathy are the thickness of the basement membrane and mesangial expansion in glomeruli, followed by glomerular sclerosis and a decrease in renal function.^{5,6}

Midkine (MK) is a heparin-binding growth factor that was first discovered as the product of a retinoic acid-responsive gene.^{7,8} The major biological roles of MK can be categorized into three areas, namely the nervous system, cancer, and inflammation.⁹ MK supports neuronal cell survival and neurite extension.^{10,11} The neuronal cytoprotective effect of MK has been demonstrated in various *in vivo* models, including retinal degeneration induced by constant light exposure,¹² cerebral infarction,¹³ and ischemia-induced neuronal death.^{14,15} In human carcinomas, MK expression is often elevated and is associated with a poor prognosis. MK antisense oligodeoxynucleotide (ODN) can suppress not only MK expression but also tumor formation.¹⁶ In addition, MK is involved in inflammation, as revealed by studies involving MK-deficient (*Mdk*^{-/-}) mice. Arterial restenosis,¹⁷ ischemic reperfusion-induced renal damage,¹⁸ *cis*-platin-induced renal damage,¹⁹ and rheumatoid arthritis²⁰ are manifested to a lesser extent in *Mdk*^{-/-} mice. MK antisense ODN can suppress arterial restenosis,²¹ ischemic reperfusion-induced renal damage,²² and *cis*-platin-induced renal damage in *Mdk*^{+/+} mice.¹⁹

Although MK plays a critical role in the development of inflammation-related, acute renal damage,^{18,22} its role in chronic renal diseases and glomerular damage, in which

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inflammation may not play a central role, has been uncertain. In the present study, we found that MK was induced in the glomerular mesangium in diabetic nephropathy. We report here an unexpected, significant difference in diabetic nephropathy between wild-type (*Mdk*+/-) and MK-deficient (*Mdk*-/-) mice. This study reveals a novel key molecule in diabetic nephropathy.

Materials and Methods

Animals and Experimental Design

Mice deficient in the MK gene (*Mdk*) were generated as described previously.²³ After backcrossing of *Mdk*+/- mice for 14 generations with 129/SV mice, *Mdk*+/- mice were mated with each other to generate *Mdk*+/+ and *Mdk*-/- mice, which were used in this study. Experiments were performed on 8- to 12-week-old female mice weighing 20 to 25 g that were housed under controlled environmental conditions and maintained with standard food and water.

Diabetes mellitus was induced in *Mdk*+/+ mice and *Mdk*-/- mice with single intraperitoneal injections of Streptozotocin (STZ; 140 mg/kg body weight; Sigma, St. Louis, MO) on 2 consecutive days. The blood glucose levels were measured weekly after injection of STZ using a glucometer. Mice with blood glucose levels of more than 250 mg/dl were regarded as being in a diabetic state. The levels were maintained between 350 mg/dl and 500 mg/dl within the experimental period. Mice were sacrificed at 2, 4, and 6 months after injection of STZ. Kidneys were removed for examination. Blood and urine samples were collected on the day of sacrifice. Kidney tissues were processed for histology and protein extraction. All of the animal experiments were performed in accordance with the animal experimentation guide of Nagoya University School of Medicine.

Biochemical Examination

Renal function was evaluated by determination of urine-albumin/urine-creatinine, urine-protein/urine-creatinine, and blood urea nitrogen (BUN). Albumin, protein, and creatinine in urine, and BUN were measured using a Mouse Albumin enzyme-linked immunosorbent assay (ELISA) kit (Shibayagi, Gunma, Japan), a micro-TP test kit (Wako, Osaka, Japan), a Cre-Kainos kit (Kainos Co., Ltd., Tokyo, Japan), and an Iatrochrom UN Kit (Iatron Co., Ltd., Tokyo, Japan), respectively.

Morphological Assessment

The removed kidneys were fixed in 4% paraformaldehyde, embedded in paraffin and then cut into 4- μ m sections. The sections were stained with periodic acid-Schiff reagent (PAS) and periodic acid-methenamine-silver (PAM). For the morphometric analysis, the extent of glomerular sclerosis was assessed by examining 20 glomeruli cut at their vascular poles in a section. The extent of increase in mesangial matrix was determined by assess-

ing the PAS-positive and nuclei-free area in the mesangium.^{24,25} The glomerular area was also treated along the outline of capillary loop. Their areas were measured using MetaMorph 6.3 image analysis computer program (Universal Imaging Co., West Chester, PA).^{26,27}

Immunohistochemical Labeling for Type I and IV Collagen, Macrophages, and Midkine

Parts of the kidney tissues were snap-frozen in liquid nitrogen. Sections (4- μ m thick) were cut with a cryostat and fixed in acetone. The sections were stained with polyclonal rabbit anti-rat type I collagen (Chemicon International, Temecula, CA) or rabbit anti-bovine type IV collagen (LSL Co., Tokyo, Japan), followed by detection with fluorescein isothiocyanate (FITC) goat anti-rabbit IgG (Zymed Laboratories, San Francisco, CA).

Cryosections were stained with monoclonal rat anti-mouse monocyte-macrophage marker F4/80 (Serotec, Oxford, UK), followed by detection with FITC rabbit anti-rat IgG (Zymed Laboratories). Leukocytes positive for F4/80 were counted by examining 20 glomeruli under a microscope at 400 $\times$ magnification in a blind manner.¹⁸

Tissue sections were pretreated with 0.4% hydrogen peroxide for 20 minutes, followed by avidin and biotin block (Vector Laboratories, Burlingame, CA) and 10% normal rabbit serum for 1 hour to prevent nonspecific detection. Sections were then incubated with chicken anti-human MK overnight at 4°C and biotin-conjugated rabbit anti-chicken IgG (ICN Pharmaceuticals Inc., Aurora, OH) for 1 hour. Immunostaining was performed by the streptavidin (Chemicon International)-biotin immunoperoxidase method. The staining was visualized with 3,3'-diaminobenzidine (Dako, Carpinteria, CA) to produce a brown color.

Cryosections were stained with chicken anti-human MK overnight at 4°C, followed by detection with FITC goat anti-chicken IgG (Zymed Laboratories) for 1 hour. Negative controls were performed by replacing primary antibodies with species-matched antibodies.

Cell Culture

A glomerular mesangial primary culture was established from glomeruli isolated by conventional sieving methods from the kidneys of adult *Mdk*+/+ or *Mdk*-/- mice and was identified according to the method previously described.^{28,29} Mesangial cells between the fourth and sixth passages were plated on 60-mm plastic dishes (Iwai Chemicals, Tokyo, Japan) and maintained in growth medium (3:1 mixture of DMEM:Ham's F-12 medium supplemented with 1 mmol/L glutamine, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 20% fetal bovine serum; GIBCO-BRL, Gaithersburg, MD).³⁰ Murine glomerular mesangial cells (MES-13) were obtained from the American Type Culture Collection (Manassas, VA) and maintained in DMEM containing 5.4 mmol/L D-glucose supplemented with 5% fetal bovine serum. Subconfluent mesangial cells were incubated in serum-free medium for 24 hours to arrest and synchronize cell growth. After this

Table 1. Blood Glucose and Body Weight in *Mdk*^{+/+} and *Mdk*^{-/-} Mice

	Pre-injection	2 month	4 month	6 month
Blood glucose (mg/dl)				
<i>Mdk</i> ^{+/+}	120 ± 13.6	424 ± 54.1	398 ± 53.7	388 ± 111
<i>Mdk</i> ^{-/-}	119 ± 19.5	395 ± 59.8	370 ± 56.8	379 ± 87.7
Body weight (g)				
<i>Mdk</i> ^{+/+}	21.1 ± 1.8	20.7 ± 2.5	23.7 ± 2.6	25.6 ± 4.6
<i>Mdk</i> ^{-/-}	20.7 ± 1.8	19.8 ± 1.2	22.3 ± 2.9	24.1 ± 2.5

Data are means ± SEM (*n* = 12).

period, the medium was changed to fresh serum-free DMEM containing 25 mmol/L D-glucose (Sigma) or DMEM containing 5.4 mmol/L D-glucose (Sigma) for 0, 3, 6, 12, or 24 hours. GF109203X (Sigma) was used as a PKC inhibitor to confirm the relationship between MK and PKC. In another experiment, cells were also exposed to 25 mmol/L D-glucose medium in the presence of 100 ng/ml human recombinant MK for 0, 3, 6, 12, or 24 hours.³¹ Cells were then lysed in RIPA buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% Nonidet P-40, 1% deoxycolic acid, and 0.05% sodium dodecyl sulfate) containing 0.25 mmol/L phenylmethylsulfonyl fluoride, kept on ice for 60 minutes, and then centrifuged at 15,000 × *g* for 10 minutes at 4°C. The supernatants were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting.

Western Blot Analysis

Mouse kidney tissues were snap-frozen in liquid nitrogen for protein isolation. Western blot analysis was performed as described previously.³² The blots were subsequently incubated with rabbit anti-MK antibody, monoclonal anti-β-actin antibody (Sigma-Aldrich, St. Louis, MO), rabbit anti-phospho-p44/42 mitogen-activated protein (MAP) kinase polyclonal antibody, rabbit anti-phospho-protein kinase C (pan) antibody (Cell Signaling Technology, Beverly, MA), or mouse anti-pan-ERK (Transduction Laboratories, Lexington, KY), followed by incubation with peroxidase-conjugated rabbit IgG and mouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Proteins were visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia, Amersham Biosciences, Piscataway, NJ). The density of each band was measured using the public domain NIH Image program.

TGF-β₁ Protein ELISA Assay

After cell growth had been synchronized, subconfluent mesangial cells were incubated in fresh DMEM containing 25 mmol/L D-glucose and 0.5% fetal bovine serum. Cell culture supernatants were collected after a certain time, and TGF-β₁ levels were measured using an ELISA kit (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. The results were normalized as to the total protein concentration.

In Vitro Administration of Antisense ODN Targeted to MK

MES-13 cells were transfected with mouse MK antisense or scramble ODN as described previously.^{16,22} After 48 hours of incubation in serum-free DMEM containing 25 mmol/L D-glucose, conditioned medium was collected for analysis of MK expression, and a cell lysate was used for evaluation of phosphorylated ERK 1/2.

Statistical Analysis

All values are expressed as means ± SEM. Statistical analysis was performed with unpaired, two-tailed Student's *t*-test for single comparisons or analysis of variance for multiple comparisons. A *P* value of <0.05 was taken to indicate a significant difference.

Results

Body Weight and Blood Glucose Levels

To develop a mouse model of diabetes mellitus, we injected STZ into *Mdk*^{+/+} or *Mdk*^{-/-} mice with the 129sv background. Insulin treatment was not performed. Blood glucose was markedly increased 2 months after STZ administration (Table 1). There was not an apparent body weight loss (Table 1). There were no significant differences in body weight and blood glucose levels between *Mdk*^{+/+} and *Mdk*^{-/-} mice (Table 1).

Glomerular Sclerosis Is Less Marked in MK-Deficient Mice

To determine the role of MK in the chronic phase of diabetic nephropathy *in vivo*, we compared the degrees of glomerular sclerosis in *Mdk*^{+/+} and *Mdk*^{-/-} mice. No obvious mesangial expansion was observed by light microscopy in either genotype a month after STZ injection, although the glomerulus became hypertrophic (data not shown). At 2 months after STZ injection, *Mdk*^{+/+} mice exhibited a diffuse increase in PAS-positive materials in the mesangial areas (Figure 1A, b), which became more diffuse and severe at 4 months (Figure 1A, c). At 6 months, the glomeruli of *Mdk*^{+/+} mice showed diabetic nodular lesions (Figure 1A, d), as demonstrated by thick-

Figure 1. Glomerular histology of STZ-induced nephropathy in *Mdk*^{+/+} and *Mdk*^{-/-} mice. **A:** A glomerulus at 0, 2, 4, and 6 months, respectively, is shown by PAS staining. **a–d:** *Mdk*^{+/+} mice; **e–h:** *Mdk*^{-/-} mice. Bar = 50 μ m. **B:** A glomerulus at 6 months is shown by PAM staining. **a:** *Mdk*^{+/+} mice; **b:** *Mdk*^{-/-} mice. Bar = 50 μ m.

ening of the glomerular basement membrane of the peripheral capillary loops and the lobular formation of diabetic nodular lesions observed on PAM staining (Figure 1B, a). By contrast, *Mdk*^{-/-} mice showed only a few PAM-positive areas at 6 months (Figure 1B, b). After 4 months, the glomeruli of *Mdk*^{+/+} mice were gradually reduced in size and finally became obsolescent glomeruli (Figure 1A, d). It is of note that the increase in the mesangial matrix was not associated with an increase in the number of nuclei (Figure 1). Overall, the histological features of *Mdk*^{+/+} mice indicated severe glomerular sclerosis. Diffuse obsolescent glomeruli were found 1

Figure 2. Morphometric analysis of glomerular pathology in *Mdk*^{+/+} and *Mdk*^{-/-} mice. **A:** Glomerular area (μ m²). **B:** Mesangial area (μ m²). **C:** Mesangial area/glomerular area. Data are shown as means (columns) and SEM (bars). $\square$, *Mdk*^{+/+} mice; $\blacksquare$, *Mdk*^{-/-} mice. * P < 0.0001; ** P < 0.005. n = 12.

year after STZ injection in *Mdk*^{+/+} mice (data not shown).

The histological observation shown in Figure 1A was further evaluated quantitatively with the aid of an image analysis program. Glomerular area of *Mdk*^{-/-} mice increased with age, whereas that of *Mdk*^{+/+} mice transiently increased at 2 months and then decreased afterward (Figure 2A). Mesangial area and mesangium-to-glomeruli ratio increased in both genotypes but

significantly more in *Mdk*^{+/+} than *Mdk*^{-/-} mice (Figure 2, B and C).

Renal Function

Renal function was then evaluated by means of urine-albumin/urine-creatinine, urine-protein/urine-creatinine and BUN values. Both microalbuminuria, which is a hallmark of glomerular hyperfiltration, and overt proteinuria are good predictors of the development of diabetic nephropathy. Both genotypes showed microalbuminuria to a similar extent at 2 weeks, but microalbuminuria of *Mdk*^{+/+} mice strikingly increased after 4 weeks (Figure 3A). Consequently, microalbuminuria was significantly more severe in *Mdk*^{+/+} than *Mdk*^{-/-} mice between 4 and 8 weeks (Figure 3A). Proteinuria became apparent from 2 months in both genotypes; the levels were much higher in *Mdk*^{+/+} than *Mdk*^{-/-} mice (Figure 3B). BUN gradually increased toward 6 months in both genotypes. *Mdk*^{+/+} mice showed significantly higher BUN levels than *Mdk*^{-/-} mice after 4 months (Figure 3C).

The Accumulation of Type I and IV Collagen

Type I and IV collagens are the PAS-positive materials in the mesangial area, and deposition of them is a hallmark of glomerular sclerosis. Both type I and IV collagen-stained areas increased in *Mdk*^{+/+} and *Mdk*^{-/-} mice at 4 and 6 months, but to higher extents in *Mdk*^{+/+} mice (Figure 4).

MK Expression during the Development of Glomerular Sclerosis

We next addressed whether MK expression was increased *in vivo*, particularly in the glomerulus, during the pathogenesis of diabetic nephropathy. Strong staining of MK protein in the glomerulus was detected in the *Mdk*^{+/+} mice treated with STZ (Figure 5, A and B). MK staining became apparent at 2 months (Figure 5A (b) and 5B (b)), and nodular and stronger at 4 and 6 months (Figure 5A, c and d). The overall profile was reminiscent of those of PAS staining and collagen I and IV deposition (Figures 1 and 4). In addition to changes in the glomerulus, increase of MK expression was also observed in tubules at 4 months (Figure 5C).

We then attempted to determine the direct effect of glucose on MK expression in the glomerulus. Exposure of primary cultured mesangial cells from *Mdk*^{+/+} mice to 25 mmol/L D-glucose led to a significant increase in the cellular expression of MK protein, which reached the maximum level at 6 hours (Figure 6, A and B). MK expression was similarly enhanced in the mesangial cell line MES-13 when exposed to 25 mmol/L D-glucose (Figure 6C). D-Glucose, but not the enantiomer L-glucose, acts as an energy source and causes diabetes mellitus if present at high concentrations. Therefore, L-glucose can be used as a negative control for "high glucose" loading.

Figure 3. Renal function in STZ-treated *Mdk*^{+/+} and *Mdk*^{-/-} mice. **A:** Urine-albumin/urine-creatinine. Urinary albumin was measured at 0, 2, 4, 6, and 8 weeks. **P* < 0.05. *n* = 5. **B:** Urine-protein/urine-creatinine. **P* < 0.001. *n* = 12. **C:** Blood urea nitrogen. Data are means ± SEM. □, *Mdk*^{+/+} mice; ■, *Mdk*^{-/-} mice. **P* < 0.001. *n* = 12.

Cells exposed to 25 mmol/L L-glucose did not show a marked increase in MK expression (Figure 6, A and D), suggesting that MK induction is due to D-glucose rather than high osmotic pressure.

Figure 4. Immunohistochemical analysis of ECM accumulation in STZ-treated *Mdk*^{+/+} and *Mdk*^{-/-} mice. Immunofluorescence staining for type I collagen (**A**) and type IV collagen (**B**). Bar = 50 μ m.

PKC and MAP Kinase Pathways

High glucose significantly increased the phosphorylation of PKC in mesangial cells, which was maximal at 6 hours after treatment (Figure 7, A and B). Mesangial cells from *Mdk*^{+/+} mice responded to high glucose more strikingly than those from *Mdk*^{-/-} mice (Figure 7, A and B). It is of note that MK expression and the phosphorylation of PKC showed similar profiles after high glucose treatment in *Mdk*^{+/+} mice (Figures 6B and 7B). However, 10 μ mol/L GF109203X, a PKC inhibitor, did not affect MK expression (Figure 7C).

Phosphorylation of ERK in mesangial cells decreased transiently and then significantly increased after exposure to high glucose (Figure 7, D and E). The phosphorylation after treatment was significantly higher at 6, 12, and 24 hours in mesangial cells from *Mdk*^{+/+} mice than in ones from *Mdk*^{-/-} mice (Figure 7, D and E). L-Glucose (25 mmol/L) and D-glucose (5.4 mmol/L) had no effect on the phosphorylation of ERK 1/2 or PKC (data not shown).

To determine whether the difference in ERK phosphorylation between *Mdk*^{+/+} and *Mdk*^{-/-} mice is due to the presence or absence of MK, we next examined the effect of MK protein on *Mdk*^{-/-} mesangial cells. In *Mdk*^{-/-} mesangial cells exposed to 25 mmol/L D-glucose in the presence of 100 ng/ml MK, ERK 1/2 phosphorylation was induced with a profile similar to that in *Mdk*^{+/+} cells exposed to 25 mmol/L D-glucose (Figure 8, A and B). Importantly, exogenous MK protein administration together with high glucose had no effect on PKC phosphorylation in *Mdk*^{-/-} mesangial cells (data not shown). To further confirm the importance of MK in ERK phosphorylation, MES-13 cells were transfected with MK antisense ODN and then exposed to 25 mmol/L D-glucose. As shown in Figure 8, C and D, MK antisense but not control

scramble ODN significantly suppressed MK production and simultaneously ERK phosphorylation.

TGF- β_1 Expression

TGF- β_1 plays an important role in the progression of diabetic nephropathy. TGF- β_1 stimulates extracellular matrix synthesis in mesangial cells through the up-regulation of phosphorylated ERK 1/2. To further prove the importance of MK in this model, we verified TGF- β_1 expression in *Mdk*^{+/+} or *Mdk*^{-/-} mesangial cells exposed to 25 mmol/L D-glucose. ELISA assaying revealed that there was no obvious difference in TGF- β_1 expression between *Mdk*^{+/+} and *Mdk*^{-/-} cells on days 1 and 2, but it was significantly increased more in *Mdk*^{+/+} cells than in *Mdk*^{-/-} cells on days 3 and 4 (Figure 9).

Macrophage Infiltration into Glomeruli

Finally, we examined macrophage infiltration. Macrophage infiltration is another key event during the pathogenesis of diabetic nephropathy. Macrophage infiltration into glomeruli was more significant in *Mdk*^{+/+} mice than *Mdk*^{-/-} mice, the maximum level being reached at 2 months (Figure 10).

Discussion

Maintenance of normoglycemia by treatment such as pancreas transplantation can induce regression of glomerular pathological changes.³³ Several cohort studies have also clearly linked longstanding hyperglycemia to diabetic nephropathy.^{34–36} Therefore, understanding of

Figure 5. MK expression in glomeruli and tubulointerstitium of STZ-treated *Mdk*^{+/+} mice. **A:** Immunohistochemical staining of glomeruli is shown. **a:** Pre-injection; **b:** 2 months; **c:** 4 months; **d:** 6 months. **B:** Immunofluorescence staining of glomeruli. **a:** Pre-injection; **b:** 2 months. **C:** Immunohistochemical staining of tubulointerstitium. **a:** Pre-injection; **b:** 4 months. Bar = 50 μ m.

the molecular events evoked by hyperglycemia is important for therapeutic strategies for diabetic nephropathy. Among such molecular events, activation of ERK is a critical one. Thus, ERK phosphorylation is linked to overproduction of TGF- β , which is considered to be the final mediator of ECM accumulation and hyperfiltration in this disease.³⁻⁵ We demonstrated here that MK is induced by high glucose. ERK phosphorylation on high glucose is less in *Mdk*^{-/-} mesangial cells and is enhanced by the combination of high glucose and exogenous MK. MK knockdown reduces ERK phosphorylation. Our data strongly suggest that MK accelerates the intracellular signaling network, particularly via ERK activation, evoked by longstanding hyperglycemia.

Because high glucose activates PKC and ERK and the general PKC inhibitor calphostin C can prevent ERK activation with high glucose,^{3-5,37} ERK and PKC are thought to be tightly associated. We demonstrated here that the combination of exogenous MK and high glucose only induces ERK phosphorylation, ie, not PKC phosphoryla-

Figure 6. MK expression in cultured mesangial cells from *Mdk*^{+/+} mice. **A:** Time course of MK expression in cultured mesangial cells on exposure to 25 mmol/L D-glucose or L-glucose. MK protein was determined by Western blotting. **B:** The intensity of MK bands obtained from D-glucose treatment in **A** was normalized as to β -actin. Data are means $\pm$ SEM for three independent experiments. * P < 0.05. **C:** Time course of MK expression in MES-13 cells on exposure to 25 mmol/L D-glucose. **D:** MK expression in cultured mesangial cells on exposure to 25 mmol/L D-glucose, 25 mmol/L L-glucose, or 5.4 mmol/L D-glucose for 6 hours. The intensity of MK bands was normalized as to β -actin. Data are means $\pm$ SEM for three independent experiments. * P < 0.05 versus 5.4 mmol/L D-glucose.

Figure 7. Comparison of glucose-induced signal pathways in cultured *Mdk*^{+/+} and *Mdk*^{-/-} mesangial cells. **A:** Cultured *Mdk*^{+/+} and *Mdk*^{-/-} mesangial cells were examined for the expression of phosphorylated PKC after 25 mmol/L D-glucose exposure. **B:** The intensity of phospho-PKC bands in **A** was normalized as to β -actin. Data are means $\pm$ SEM for three independent experiments. **P* < 0.01; ***P* < 0.001; #*P* < 0.0001 versus *Mdk*^{+/+} mesangial cells at 0 hour. $\square$, *Mdk*^{+/+}; $\blacksquare$, *Mdk*^{-/-}. **C:** Cultured mesangial cells were examined for the expression of phosphorylated PKC and MK after 6 hours exposure to 25 mmol/L D-glucose and 10 μ mol/L GF109203X. **D:** The phosphorylation of ERK 1/2 (p44/42 MAP kinase) was examined in cultured mesangial cells from *Mdk*^{+/+} mice after 25 mmol/L D-glucose exposure. **E:** The quantitative results in **D** were expressed as the relative ratio of phosphorylated to pan-ERK. Data are means $\pm$ SEM for three independent experiments. $\square$, *Mdk*^{+/+}; $\blacksquare$, *Mdk*^{-/-}. **P* < 0.001; ***P* < 0.0001. #*P* < 0.001 versus *Mdk*^{+/+} mesangial cells at 0 hour.

tion, in *Mdk*^{-/-} mesangial cells. Our data indicate that MK is necessary but not sufficient for PKC activation, whereas it is necessary and sufficient for ERK phosphorylation. Therefore, this study provides a model for the

Figure 8. Effect of MK on the phosphorylation of ERK 1/2 in cultured mesangial cells from *Mdk*^{-/-} mice and MES-13 cells under high glucose conditions. **A:** Phosphorylated ERK expression in *Mdk*^{-/-} mesangial cells exposed to 100 ng/ml MK was almost equal to that in *Mdk*^{+/+} under high glucose conditions. **B:** The phosphorylated/pan-ERK ratios in **A** are shown. The results are means $\pm$ SEM for four independent experiments. **P* < 0.0001 versus *Mdk*^{-/-} without MK exposure at each time point under high glucose conditions. $\square$, *Mdk*^{+/+}; $\blacksquare$, *Mdk*^{-/-}. **C:** MES-13 cells were transfected with antisense or scramble ODN and then examined for the expression of phospho-ERK 1/2 after 24 hours exposure to 25 mmol/L D-glucose. AS, MK antisense ODN; Scr, MK scramble ODN; NT, no treatment. **D:** The intensity of phospho-ERK 1/2 bands in **C** was normalized as to pan-ERK. Data are means $\pm$ SEM for three independent experiments. **P* < 0.01 versus MK scramble ODN.

study of molecular mechanisms differentiating these two general intracellular signaling molecules. Supporting our study, it has been reported that p8 is activated under the control of ERK and plays a critical role in mesangial hypertrophy.³⁸

Figure 9. TGF- β_1 expression in *Mdk*^{+/+} or *Mdk*^{-/-} mesangial cells under high glucose conditions. TGF- β_1 was measured by ELISA. Data are means $\pm$ SEM for five independent experiments. $\square$, *Mdk*^{+/+}; $\blacksquare$, *Mdk*^{-/-}. * $P < 0.05$; ** $P < 0.01$.

Based on the present data, we are currently considering the molecular network in diabetic nephropathy, as illustrated in Figure 11. Besides PKC, ERK, and TGF- β , the infiltration of macrophages into the glomerulus is critical in the early phase of the pathogenesis of diabetic nephropathy.^{39,40} MK may also contribute to the macrophage infiltration in this disease. This idea is supported by data that the glomerular infiltration of macrophages was reduced in *Mdk*^{-/-} mice in diabetic nephropathy. Induction of macrophage infiltration by MK has been established in several *in vivo* and *in vitro* models.^{17,18,20,41} In addition, induction of MK expression in renal tubules in our study suggest that pathological changes in the inter-

Figure 11. Schematic diagram showing the possible relationship between MK- and glucose-induced signaling pathways that promote glomerular sclerosis.

stitium and tubule also contribute to the pathogenesis of diabetic nephropathy.

There are currently ongoing phase 3 clinical trials with a PKC inhibitor. However, one should be very careful about adverse effects because ERK and PKC are general signaling molecules. In this context, it is of note that MK is expressed at low levels in adult tissues and only induced in some pathological conditions, such as carcinogenesis,^{42–44} inflammation,^{17–20,41} and diabetic nephropathy. Thus, MK is an intriguing candidate molecular target for a therapy for diabetic nephropathy. Several knockdown studies, involving such as treatment of carcinomas,¹⁶ arterial restenosis,²¹ and reperfusion renal injury,²² demonstrated that MK could be an appropriate target without apparent adverse effects.

Several models of diabetic nephropathy have been reported.^{4,24–27,45} However, in most cases, it is hard to obtain glomerular sclerosis even when hyperglycemia has been achieved. We thus considered the effects of the genetic backgrounds of the mouse strains used. As to glomerular sclerosis, a report has demonstrated that 129/SV is prone to glomerular sclerosis compared with C57BL/6J in a 5/6 kidney removal model.⁴⁶ In the present study, we succeeded in establishing glomerular sclerosis after hyperglycemia induced by injection of STZ. Our model will be useful for further study on the molecular mechanisms underlying diabetic nephropathy.

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Figure 10. Macrophage infiltration in STZ-treated *Mdk*^{+/+} and *Mdk*^{-/-} mice. **A:** Immunofluorescence staining with F4/80 antibody of glomeruli at 2 months. **a:** *Mdk*^{+/+} mice; **b:** *Mdk*^{-/-} mice. **Arrows** indicate some of the positively stained cells. Bar = 50 μ m. **B:** The numbers of macrophages that had infiltrated into the glomeruli at 2 and 4 months were determined. They were scored by examining 20 glomeruli under a microscope at 400 $\times$ magnification. Data are means $\pm$ SEM. $\square$, *Mdk*^{+/+}; $\blacksquare$, *Mdk*^{-/-}. * $P < 0.01$, $n = 12$.

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