Growth arrest-specific gene 6 is involved in glomerular hypertrophy in the early stage of diabetic nephropathy

Kojiro Nagai¹, Hidenori Arai¹, Motoko Yanagita¹, Takeshi Matsubara¹, Hiroshi Kanamori¹, Toru Nakano⁴, Noriyuki Iehara³, Atsushi Fukatsu³, Toru Kita², and Toshio Doi⁵

¹Department of Geriatric Medicine, ²Cardiovascular Medicine, and ³Artificial Kidneys, Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan

⁴Discovery Research Laboratory, Shionogi Co., Ltd., Osaka 553-0002, Japan

⁵Department of Clinical Biology and Medicine, Tokushima University, Tokushima 770-8503, Japan

Running title: Gas6 in diabetic nephropathy

Address reprint requests to H. Arai, M.D., Ph.D., Department of Geriatric Medicine, Kyoto University Graduate School of Medicine, 54 Kawahara-cho, Shogoin, Sakyō-ku, Kyoto 606-8507, Japan. Tel: 81-75-751-3463, fax: 81-75-751-3463, e-mail: harai@kuhp.kyoto-u.ac.jp
Abstract

Nephropathy is one of the most common complications of diabetes mellitus. Glomerular hypertrophy is a hallmark in the early phase of the nephropathy. The mechanism of glomerular hypertrophy, however, remains incompletely understood. We have reported that growth arrest-specific gene 6 (Gas6) and its receptor, Axl play a key role in the development of glomerulonephritis. Here we show the important role of Gas6/Axl in the pathogenesis of diabetic glomerular hypertrophy. In streptozotocin (STZ)-induced diabetic rats, mesangial and glomerular hypertrophy and an increase in the glomerular filtration rate (GFR) and albuminuria were observed after 12 weeks of STZ injection. The glomerular expression of Gas6 and Axl was increased in those rats. Administration of warfarin inhibited mesangial and glomerular hypertrophy and the increase in GFR and albuminuria in STZ-rats. Moreover, we found less mesangial hypertrophy in STZ-treated Gas6 knockout mice than control mice. In vitro we found that stimulation of mesangial cells with Gas6 resulted in mesangial cell hypertrophy. Thus we have found a novel mechanism of glomerular hypertrophy through the Gas6/Axl-mediated pathway in the development of diabetic nephropathy. Inhibition of the Gas6/Axl pathway in diabetic patients might be beneficial to slow down the progression of diabetic nephropathy.
Introduction

Diabetes is the most common cause of end-stage renal disease in many countries. Approximately 30% of type 1 diabetic patients suffer from diabetic nephropathy (1,2). Therefore, tremendous efforts have been made to elucidate the molecular mechanism of diabetic nephropathy to develop the effective treatment. The feature characteristic of diabetic nephropathy is persistent albuminuria and mesangial expansion followed by glomerulosclerosis and a decline in renal function. The development of glomerulosclerosis in diabetes mellitus is always preceded by the early hypertrophic processes in the glomerular compartment (3). Since it is important to regulate the early stage of the disease process, extensive efforts have been made to elucidate growth factors and cytokines involved in mesangial expansion or hypertrophy (4). Transforming growth factor-β1 (TGF-β1) and angiotensin II are found to be implicated in the development of diabetic nephropathy among them (5). Although angiotensin converting enzyme inhibitors and/or type I angiotensin receptor blockers are effective to some extent, angiotensin converting enzyme inhibitors can decrease the risk of developing diabetic nephropathy by only 12.5% in type 2 diabetic patients (6). Therefore, additional pathogenetic mechanisms are urgently investigated to help design novel therapies for patients with diabetic nephropathy.

Among other potential growth factors for glomerular cells, we have investigated the role of Gas6 in the pathogenesis of kidney disease. Gas6, cloned from serum-starved fibroblasts (7), is...
posttranslationally activated by γ-carboxylation of glutamate residues at its N terminus in the presence of vitamin K and inhibited by the anticoagulant warfarin (8,9). Recently we showed that Gas6 is an autocrine growth factor for mesangial cells, and that Gas6 and its receptor Axl play a critical role in the development of glomerulonephritis by showing that warfarin and the extracellular domain of Axl inhibit mesangial cell proliferation by specific blockade of the Gas6-mediated pathway in a mesangial proliferative model of glomerulonephritis (10,11). However, the role of Gas6 and Axl in diabetic nephropathy is not determined.

The present study is designed to examine whether Gas6 and Axl can contribute to the pathogenesis of diabetic glomerular hypertrophy in vivo and in vitro. In this study, we specifically asked whether Gas6 and Axl can play an important role in mesangial cell hypertrophy, which is a feature seen in the early phase of diabetic nephropathy and whether inhibition of the Gas6/Axl pathway can affect the progression of diabetic nephropathy in streptozotocin (STZ)-rats and mice.

**Experimental procedures**

**Materials**

STZ was obtained from Wako Pure Chemical Inc. Ltd. (Osaka, Japan). Recombinant human TGF-β1 was purchased from R&D systems, Inc. (Minneapolis, MN).

**Animals and Cell Culture**

Male Sprague-Dawley rats weighing 170-200 g were purchased from Shimizu Laboratory Animal
Gas6 knockout mice were generated with a targeted disruption of the Gas6 gene as previously described (11). Control inbred C57BL/6 mice were obtained from Clea Japan, Inc. (Osaka, Japan). Animals were housed under specific pathogen-free conditions at the Animal Facilities of Kyoto University, Faculty of Medicine. All animal experiments were performed in accordance with institutional guidelines, and the Review Board of Kyoto University granted an ethical permission to this study. Glomerular mesangial primary culture was established from glomeruli isolated from normal 4 week-old mice (C57BL/6JxSJL/J) and was identified according to the method previously described (12,13). Phenotypically stable mesangial cells, passage 12th to 16th, were plated on 100-mm plastic dishes (Nalge Nunc International, Roskilde, Denmark) and maintained in growth medium (3:1 mixture of Dulbecco’s modified Eagle’s medium/F12 modified trace elements) (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 1 mM glutamine, penicillin at 100 units/ml, streptomycin at 100 µg/ml (Invitrogen Corporation, Carlsbad, CA), and 20% fetal bovine serum (Cansera International Inc., Canada).

**STZ-induced diabetic rats and mice**

Male rats weighing 170-200 g were made diabetic by a single intravenous injection of STZ (55 mg/kg body weight) in 0.05 mol/L citrate buffer (pH 4.5). Weight-matched 8 week-old mice (17-20 g) were made diabetic by two consecutive daily intraperitoneal injections of STZ (150 mg/kg).
dissolved in 0.01 mol/L citrate buffer. Rats and mice receiving an injection of citrate buffer were used as controls. The levels of blood glucose were determined 2 days after injection of STZ or vehicle, and rats and mice with blood glucose levels more than >16.7 mmol/l were used as diabetic (14,15). Twelve weeks after STZ-injection, rats and mice were weighed and sacrificed.

Warfarin treatment

Rats were divided into 4 groups; control rats without treatment, control rats with warfarin treatment, diabetic rats without treatment, and diabetic rats with warfarin treatment. Rats with warfarin treatment were administered with 0.25 mg/L warfarin potassium (provided by Eisai Co. Ltd., Tokyo, Japan) in drinking water from the day of STZ injection. The dosage of warfarin was determined based on the previous report (10). Because diabetic rats drink much more water, the dosage of warfarin was reduced to 0.06 mg/L from 2 days after injection of STZ. Twelve weeks after injection, rats were weighed and sacrificed. Blood was collected at sacrifice. Prothrombin times, hematocrits, serum creatinine, and plasma concentrations of warfarin were measured as described (10). HbA1c was measured using DCA2000 analyzer (Bayer Medical, Tokyo, Japan). Before sacrifice, creatinine and albumin were measured from 24-hour urine collection.

Immunohistochemistry

Kidney tissues from each animal were snap frozen in cold acetone in OCT compound (Sakura Finetechnical Co. Ltd., Tokyo, Japan) and cryostat sections (4 µm) were stained by indirect...
immunofluorescence procedure with the following primary antibodies: rabbit polyclonal antibodies against rat Gas6 (16) and human Axl (generous gift from Dr. Brian Varnum, Amgen, Thousand Oaks, CA).

Isolation of glomeruli

Glomeruli were isolated from renal cortex of rats using the differential sieving method (17,18). The purity of the glomeruli was >90%.

Western blotting

Isolated glomeruli were suspended in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 % NP40, 0.25 % SDS, 1mM Na$_3$VO$_4$, 2 mM EDTA, 1 mM PMSF, 10 $\mu$g/ml of aprotinin), and incubated for 1 h at 4°C. After centrifugation, the supernatants were used as total cell lysates. Sixty $\mu$g of each sample was applied to SDS-PAGE. After electrophoresis, the proteins were transferred to nitrocellulose filters (Schleicher & Schuell Inc., Keene, NH). The blots were subsequently incubated with rabbit anti-rat Gas6, anti-human Axl, rabbit anti-phospho-p44/p42 mitogen-activated protein (MAP) kinase polyclonal antibody (Cell Signaling Technology, Beverly, MA), or rabbit anti-MAP kinase polyclonal antibody (Oncogene, San Diego, CA), followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham Biosciences, Piscataway, NJ). Immunoreactive bands were visualized using horseradish peroxidase-conjugated secondary antibody and the enhanced chemiluminescent system (Amersham Biosciences).
Histological Examination

The mesangial cell area was measured in a hematoxylin eosin staining section by Image-Pro Plus (Media Cybernetics, Silver Spring, MD). For each animal, 50 mesangial cell areas were analyzed. Glomerular surface area and periodic acid-methenamine-silver (PAM)-positive area were determined using an image analyzer (Image Processor for Analytical Pathology; Sumitomo Chemical Co., Tokyo, Japan) (19,20). For each animal, 50 glomeruli were analyzed.

Glomerular filtration rate (GFR) and the measurement of urinary albumin excretion

Urine volume (Vu) was measured at 12 weeks in 24-hour urine collection from rats housed in an individual metabolic cage. During the urine collection, rats were allowed free access to food and water. Serum and urine creatinine concentrations (Cp and Cu) were measured and GFR was calculated by an equation; GFR = (Cu/Cp) x Vu/ Body Weight (21). The albumin concentration in the urine was measured by Nephrat (Exocell Inc., Philadelphia, PA).

[^H]-leucine incorporation and determination of cell number

Mesangial cells were plated at 1.5x10^4 cells/well in 24-well dishes. After 48 h, cells were serum-starved in Dulbecco’s modified Eagle’s medium containing 0.5% bovine serum albumin (Sigma-Aldrich Co., St.Louis, MO) for 48 h. The medium was then replaced with the fresh starving medium including various concentrations of agonist, or left untreated. After 18 h, cells were labeled with[^H]-leucine (2 μCi/ml, Amersham Biosciences) for 6 h, and the incorporation of[^H]-leucine
into acid-precipitable materials was then determined. For determination of cell number, the cells were trypsinized, resuspended in PBS, and counted with a Coulter counter Z1 (Coulter Electronics Ltd., Hialeah, FL). Data were normalized by dividing incorporation counts by the cell number and showed as fold increase over control.

**Flow cytometry**

Mesangial cells were plated at 4.5x10^5 cells/well in 100-mm plates. Cells were treated as the[^3]H]-leucine incorporation procedure. After treatment, mesangial cells were harvested by tripsinization, washed with PBS, centrifuged at 1,500 rpm for 10 min, and then resuspended in ice-cold 70% ethanol added dropwise while vortexing. Ethanol-fixed mesangial cells were then analyzed by forward light scattering on a Becton Dickinson flow cytometer (BD Biosciences, San Jose, CA).

**Statistical analyses**

Data are expressed as means ± S.D. Comparison among each group was performed by one-way ANOVA followed by Neuman-Keuls test to evaluate statistical significance between two groups. A *P* value < 0.05 was considered to be significant.

**Results**

*Expression of Gas6 and Axl in STZ-induced diabetic rats*

Our preliminary data showed that glomerular hypertrophy and an increase in GFR and albuminuria
were observed after 12 weeks of STZ injection in rats. Therefore, to examine the role of Gas6/Axl in the early phase of diabetic nephropathy in vivo, we used STZ-induced diabetic rat kidney. We analyzed the glomerulus after 12 weeks of STZ injection and found that expression of both Gas6 and Axl was significantly increased in the STZ-treated group and that they were mostly localized at endothelial and mesangial cells (Figure 1).

*Warfarin treatment inhibits induced expression of Axl and phosphorylation of p44/42 MAP kinase*

Because expression of Gas6 and Axl was induced in diabetic rats, the Gas6/Axl pathway seems to play a role in the development of diabetic nephropathy in the early phase of the disease process. Therefore, we next examined whether inhibiting this pathway can be effective in treating this experimental diabetic nephropathy. We treated rats with warfarin in drinking water as shown in figure 2. Plasma concentrations of warfarin in these rats were $0.71 \pm 0.05 \mu M$ and $0.67 \pm 0.05 \mu M$, which were significantly lower than the ordinary therapeutic concentrations as an anticoagulant. The body weight and kidney weight/body weight were not changed by warfarin treatment. Significant prolongation of prothrombin times, anemia, or bleeding tendency was not observed in all the rats during the whole period of warfarin treatment as we already found in our previous study (data not shown).

After 12 weeks of STZ injection, we isolated glomeruli from the rats and found increased glomerular expression of Gas6 and Axl by western blotting (Figure 3) as shown in figure 1. When
we treated STZ-rats with warfarin, we found that the expression of Axl was markedly inhibited in warfarin-treated STZ-rats than untreated STZ-rats. Although warfarin treatment did not affect Gas6 expression, it might be due to the fact that the antibody used for western blotting cannot discriminate active or inactive Gas6.

Since we have shown that Gas6 can activate p44/42 MAP kinase in vitro (16), we examined whether p44/42 MAP kinase can be phosphorylated in diabetic glomerular lysates and whether warfarin treatment can affect the phosphorylation. As shown in figure 3, p44/42 MAP kinase was phosphorylated in the glomerular lysates in STZ-rats and warfarin treatment abolished their phosphorylation.

*Warfarin shows a beneficial effect on mesangial and glomerular hypertrophy*

Since glomerular hypertrophy is one of the earliest structural alterations in diabetic nephropathy, we measured mesangial cell and glomerular surface areas in diabetic rat kidney and examined the effect of warfarin on glomerular hypertrophy. After 12 weeks of STZ injection, both areas were significantly enlarged compared with control rats, and administration of warfarin prevented the increase of mesangial and glomerular areas (Figure 4). Because accumulation of mesangial extracellular matrix components is also an early structural change in diabetic nephropathy, we also measured the PAM-positive area in both groups. However, there was no change in the PAM-positive area between control and diabetic groups, indicating that there is no glomerular sclerotic change.
after 12 weeks of STZ injection (data not shown).

**Warfarin treatment improves hyperfiltration and excretion of urinary albumin**

In the early phase of diabetic nephropathy, GFR is increased in most of diabetic patients. Therefore, we examined whether GFR is increased in STZ-rats and whether warfarin treatment can affect GFR. After 12 weeks of STZ injection, GFR and urinary albumin excretion were significantly increased and the increased GFR and albuminuria were suppressed by warfarin treatment (Figure 5).

**STZ-treated Gas6 knockout mice showed less glomerular hypertrophy**

To confirm the specificity of warfarin on the Gas6/Axl pathway, we used STZ-treated Gas6 knockout mice. Our preliminary data showed that glomerular hypertrophy was observed after 12 weeks of STZ injection in mice. Therefore, we analyzed mesangial and glomerular hypertrophy in Gas6 knockout and wild type mice after 12 weeks of STZ injection. As shown in Figure 6A, Gas6 knockout mice were smaller than wild type mice and HbA1c was higher in Gas6 knockout mice than in wild type mice. However, the blood glucose levels were almost the same throughout the study period (data not shown). Although the kidney weight/body weight was smaller in diabetic Gas6 knockout mice compared with diabetic wild type mice, there was no statistically significant difference. As shown in Figure 6B and 6C, mesangial cell and glomerular surface areas in diabetic wild type mice were significantly larger than those in wild type untreated mice. However, in diabetic Gas6 knockout mice, the increase of both areas was significantly suppressed. These data
also indicate that Gas6 is involved in the development of the initial phase of diabetic nephropathy and suggest that warfarin inhibits diabetic nephropathy specifically through the Gas6-mediated pathway.

*Gas6 induces mesangial cell hypertrophy in vitro*

To investigate the mechanism by which Gas6 is involved in glomerular hypertrophy in diabetic rats, we examined whether Gas6 can cause mesangial cell hypertrophy *in vitro*. We measured \[^3H\]-leucine incorporation in mouse mesangial cells as a marker of cellular hypertrophy after incubation with various concentrations of Gas6. Recombinant Gas6 increased incorporation of \[^3H\]-leucine incorporation dose-dependently, with a 1.5 fold-increase at maximum (Figure 7). The same dose of Gla-defective Gas6, which is an inactive form of Gas6 without γ-carboxylation, did not affect \[^3H\]-leucine incorporation in mouse mesangial cells. The Gas6-mediated increase in \[^3H\]-leucine incorporation was almost as the same as that of TGF-β1 (1ng/ml). To clarify whether mesangial cell hypertrophy is mediated specifically through the Gas6-Axl pathway, we used the recombinant extracellular domain of Axl (Axl-Fc), which is a recombinant fusion protein of the extracellular domain of Axl and human Fc portion, as an inhibitor of the Gas6-Axl pathway. After preincubation with 10 nM Axl-Fc in starving medium for 1h, Gas6 (100ng/ml) was added to the serum-starved mesangial cells and \[^3H\]-leucine incorporation was then measured. Addition of Axl-Fc inhibited the increased \[^3H\]-leucine incorporation by Gas6, suggesting that the effect of
Gas6 on hypertrophy is specific for the Gas6-Axl interaction. Further, we checked the cellular size of mesangial cells by flow cytometry under the same protocol as \(^{3}H\)-leucine incorporation (Figure 8). We found that treatment of the cells with Gas6 100 ng/ml or TGF-\(\beta\)1 increased the cellular size by 1.1 folds, but not Gla-defective Gas6.

**Discussion**

In this study, we have shown a novel mechanism of mesangial hypertrophy in diabetic nephropathy mediated by Gas6. This is the first demonstration that Gas6 can induce mesangial cell hypertrophy characteristic of the early stage of diabetic nephropathy and that warfarin is effective to prevent the progression of diabetic nephropathy. Our study implies that Gas6 can be a novel growth factor that plays a crucial role in the development of the initial phase of diabetic nephropathy.

Here we have found a novel aspect of warfarin as an anti-hypertrophic agent. Our data also show that warfarin treatment ameliorated hyperfiltration and urinary albumin excretion in STZ-rats. Thus hypertrophy and hyperfiltration might be an interactive mechanism and presumed to be causally linked (22). It is conceivable, therefore, that blocking the Gas6/Axl pathway can improve the vicious cycle in diabetic nephropathy. Therefore, treating diabetic patients with warfarin to prevent the nephropathy would be one of the options for the treatment. However, it should be noted of the side effect of warfarin if we treat diabetic patients with warfarin. Warfarin has long been used
as an anticoagulant to prevent thrombosis and embolism (23,24), and patients prescribed with this agent are monitored by measuring prolongation of prothrombin times to achieve its anticoagulant effect. These patients have to be cared for the risk of bleeding (25). However, the anti-hypertrophic effect of warfarin was achieved at serum concentrations of 0.7 µM, which is significantly lower than the ordinary therapeutic concentrations as an anticoagulant (4 to 5 µM) (26). The prothrombin times of rats treated with warfarin in our experiments were not significantly prolonged, and no bleeding tendency or anemia was observed (data not shown), while mesangial cell hypertrophy was significantly inhibited. Although we have shown the clear effect of warfarin on the development of diabetic nephropathy, the question remains about the specificity of the effect of warfarin. We have already shown that in mesangial cells warfarin specifically inhibits the Gas6/Axl pathway in vitro (16). To further confirm the specificity, we have treated Gas6-deficient mice (11) with STZ (15) and found that both mesangial and glomerular areas were significantly decreased in Gas6-deficient mice compared with wild type mice. Therefore, we can conclude that this effect of warfarin would be mediated specifically through the inhibition of Gas6.

Hypercoagulability in glomeruli has also been reported in diabetic nephropathy (27), which might worsen the renal function. Recently, Angelillo-Scherrer et al. reported that deficiency of Gas6 protects mice from thrombosis (28). In this study, we used a low dose of warfarin and at these concentrations we found no prolongation of prothrombin time (data not shown). However, we
already reported that even under these concentrations, warfarin can inhibit the activation of Gas6 \textit{in vitro} and \textit{in vivo} (10,16,29). It is still possible that warfarin could affect the coagulation cascades and prevent thrombotic events even at low concentrations. Therefore, improving the coagulation state would also be one of the possibilities that Gas6 can affect the development of diabetic renal disease.

The increase of extracellular matrix followed by mesangial cell hypertrophy is one of the major characteristics in diabetic nephropathy (30). Mauer et al. investigated structural-functional relationship in a cross-section of patients with type 1 diabetes (31). They found a close correlation between mesangial expansion and clinical manifestations of diabetic nephropathy. Ziyadeh et al. also showed that the development of irreversible renal changes in diabetes mellitus, such as glomerulosclerosis, is always preceded by the early hypertrophic processes in the glomerular compartment (3). In our experiment, however, we could not find excessive accumulation of extracellular matrix in STZ-rats at 12 weeks, although other investigators have reported increased deposition of extracellular matrix in a later phase of nephropathy in STZ-rats (32). Since our purpose in this study is to determine the role of Gas6 in the initial phase of diabetic nephropathy, we analyzed up to 12 weeks after STZ injection. The correlation between Gas6 and diabetic glomerulosclerosis should be evaluated by treating the rats with warfarin for a longer time or using another animal model. Treating STZ-rats with high protein diet would be another way to accelerate
the glomerulosclerosis (33) and should be tested in the future experiments.

We have already shown that Gas6 is a growth factor for mesangial cells *in vitro* and that Gas6 plays a key role in acute and chronic forms of glomerulonephritis *in vivo* (10,16,29). In those studies, we have shown that Gas6 can induce mesangial proliferation through a tyrosine kinase, Axl (11,16) and a transcription factor signal transducer and activator of transcription 3 (29). However, in this study we have clearly shown that Gas6 can induce mesangial hypertrophy *in vivo and in vitro*. Therefore, the obvious question is why Gas6 only induces mesangial hypertrophy without affecting the mesangial proliferation in this diabetic rat model. Although we determined the mesangial cell area in the kidney of diabetic rats, we did not notice that the glomerular cell number was increased in diabetic rats (data not shown). Although Young et al. reported that diabetic nephropathy may be associated with some glomerular cell proliferation, hypertrophy is the major finding of diabetic nephropathy (34). The reason remains uncertain why Gas6 can be induced in both glomerulonephritis and diabetic nephropathy and why Gas6 does not induce cell proliferation in diabetic nephropathy. It is conceivable to speculate that some other growth factor or cytokine is playing an additional role in determining the fate of mesangial cell in the disease process.

We have clearly shown that p42/44 MAP kinase was phosphorylated in the glomeruli after 12 weeks of STZ injection and that warfarin treatment abolished the phosphorylation. In case of IGF-1, Akt seems to be responsible for its hypertrophic effect in skeletal myotube (35) and
endothelin-induced hypertrophy requires activation of p42/44 MAP kinase, JNK/SAPK, and PI3-kinase pathways (36). Although we have no definite evidence to indicate the role of p42/44 MAP kinase in mesangial cell hypertrophy so far, phosphorylation of p42/44 MAP kinase might be used as a marker for the hypertrophy in diabetic nephropathy. The molecular mechanism of mesangial cell hypertrophy in diabetic nephropathy should be further clarified in the future studies.

In summary, this is the first demonstration that Gas6 and Axl are involved in the development of the initial phase of diabetic nephropathy by inducing mesangial cell hypertrophy. This is a completely novel mechanism explaining the development of diabetic nephropathy. Blocking this pathway would be beneficial to prevent the progression of nephropathy in diabetic patients.

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**Footnotes**

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1The abbreviation used are: TGF-β1, transforming growth factor-β1; Gas6, growth arrest-specific gene 6; STZ, streptozotocin; DMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin; GFR, glomerular filtration rate; MAPK, mitogen-activated protein kinase; PAM, periodic
acid-methenamine-silver.
Figure Legends

Fig. 1 Expression of Gas6 and Axl in STZ-induced diabetic rat kidney.

Kidney tissues from each animal were snap frozen in cold acetone in OCT compound. Cryostat sections (4 µm) were stained using indirect immunofluorescence procedure with anti-Gas6 or anti-Axl antibody. Original magnification, x400.

Fig. 2 Protocol of warfarin treatment (A) and physiological characteristics of control and diabetic rats with or without warfarin treatment after 12 weeks (B).

A single intravenous injection of 55 mg/kg of STZ was performed on day 1 to make diabetic rats. Rats were separated in control and diabetic groups with or without warfarin treatment. Rats with warfarin treatment (Wa) were administered with 0.25 mg/L warfarin in drinking water. Because diabetic rats drink much more water, the dosage of warfarin was reduced to 0.06 mg/L from 2 days after injection of STZ. (B) After 12 weeks of STZ injection, rats were weighed and sacrificed. Blood was taken to evaluate HbA1c and plasma concentrations of warfarin. Kidney weight was also measured.

Fig. 3 Effect of warfarin on the expression of Gas6 and Axl and expression and phosphorylation of p44/42 MAP kinase in STZ rat glomeruli.

After 12 weeks of STZ injection, rats were sacrificed and glomeruli were isolated by sieving method. The isolated glomeruli were suspended in RIPA buffer. After centrifugation, the
supernatants were used as total cell lysates. Sixty µg of each sample was analyzed by Western blotting with the antibodies indicated. Each lane represents a representative Western blot for the cell lysate from each rat. Wa; warfarin treatment.

Fig. 4 **Effect of warfarin on mesangial (A) and glomerular hypertrophy (B)**

The mesangial cell area was measured in a hematoxylin eosin staining section by Image-Pro Plus. For each animal, 50 mesangial cell areas were calculated. The glomerular surface area was determined using an image analyzer. Data are expressed as means ± S.D. (n=6 in control group and n=10 in diabetic group). *P <0.01, **P <0.05.

Fig. 5 **Effect of warfarin on hyperfiltration (A) and urinary albumin excretion (B)**.

Urine volume was measured at 12 weeks in 24-hour urine. Serum and urine creatinine concentrations were measured and GFR was calculated by an equation described in “Research design and Methods”. Albumin concentrations in the urine were measured by Nephrat. Data are expressed as means ± S.D. (n=6 in control group and n=10 in diabetic group). *P <0.01, **P <0.05.

Fig. 6 **Effect of Gas6 deficiency on mesangial and glomerular hypertrophy in STZ-treated mice**

Diabetes was induced by two consecutive daily intraperitoneal injections of STZ (150 mg/kg). Wild type (WT) and Gas6 knockout mice (KO) (12 each) were divided into two groups; untreated (Control) or STZ-treated (Diabetes). Twelve weeks after STZ-injection, HbA1c, body weight, and kidney weight were measured (A). Mesangial cell (B) and glomerular surface areas (C) were also
measured as described under “Experimental Procedures”. For each mouse, 50 mesangial cell and 
glomerular surface areas were calculated. Data are expressed as means ± S.D. (n=6 in each group).

*P <0.01, **P <0.05.

Fig. 7 [3H]-leucine incorporation in mouse mesangial cells by Gas6

Mesangial cells were plated at 1.5 x 10^4 cells/well in 24-well dishes. After 48 h, cells were 
serum-starved in DMEM containing 0.5% BSA for 48 h. Then the medium was replaced with the 
fresh starving medium including various concentrations of agonist, or left untreated. After 18 h, 
cells were labeled with [3H]-leucine (2 µCi/ml) for 6 h and the incorporation of [3H]-leucine into 
acid-precipitable materials was determined. The data were normalized by dividing incorporated 
counts with cell number and showed as fold increases over control. Values expressed are the means 
± S.D. of six independent experiments. *P <0.01.

Fig. 8 Flow cytometric analysis of mesangial cell size

Cells were treated as described in figure 7. After treatment, mesangial cells were harvested by 
tripsinization, washed with PBS, centrifuged at 1,500 rpm for 10 min, and then resuspend in 
ice-cold 70% ethanol. Ethanol-fixed mesangial cells were then analyzed by forward light scattering 
on a Becton Dickinson flow cytometer. Data are representative of six independent experiments with 
qualitatively similar changes. Thin line = control; bold line = agonist. (A); 100 ng/ml of Gas6, (B); 
100 ng/ml of Gla-defective Gas6, (C); 1 ng/ml of TGF-β1. (D) Means of forward scatter of
mesangial cells after treatment. Data are shown as fold increase over control. Values are the means ± S.D. of six independent experiments. **$P<0.05$. 
Figure 1.
Figure 2.

A  

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<td>(-)</td>
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B

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- **Gas6**
- **Axl**
- **Phospho-p44/42 MAP kinase**
- **p44/42 MAP kinase**
Figure 4.
Figure 5.
**Figure 6.**

A

<table>
<thead>
<tr>
<th></th>
<th>Number</th>
<th>Body WT (g)</th>
<th>HbA1c (%)</th>
<th>Kidney WT/BWT (g/100gs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Control</td>
<td>6</td>
<td>22.9 ± 0.9</td>
<td>3.6 ± 0.1</td>
<td>1.26 ± 0.06</td>
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<tr>
<td>KO Control</td>
<td>6</td>
<td>21.1 ± 1.0</td>
<td>4.2 ± 0.6</td>
<td>1.23 ± 0.10</td>
</tr>
<tr>
<td>WT Diabetes</td>
<td>6</td>
<td>19.6 ± 2.3</td>
<td>9.6 ± 0.3</td>
<td>2.11 ± 0.45</td>
</tr>
<tr>
<td>KO Diabetes</td>
<td>6</td>
<td>19.2 ± 1.1</td>
<td>10.4 ± 1.3</td>
<td>1.73 ± 0.15</td>
</tr>
</tbody>
</table>

B

![Graph showing mesangial cell area](image1)

C

![Graph showing glomerular surface area](image2)
Figure 7.

[Graph showing fold increase over control (cpm/cell count) for different concentrations of Gas6, Axl-Fc, Gla-defective, and TGF1. Symbols denote significant differences.]
Figure 8.

A. Cell Counts

B. Cell Counts

C. Cell Counts

D. Fold increase over control

Cont, Gas6, Gla, TGF

Mean of FSC
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