Alteration of VEGF and Angiopoietins Expressions in Diabetic Glomeruli Implicated in the Development of Diabetic Nephropathy

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Abstract

Etiologies of diabetic nephropathy are largely unknown. Recent investigations have shed light on the potent roles of growth factors in the development of diabetic nephropathy.

We investigated the glomerular expression of vascular endothelial growth factor-A (VEGF-A), Angiopoietin-1 (Ang-1) and Angiopoietin-2 (Ang-2) in diabetic condition and the effects of those factors on the glomerular endothelial gene

expression. The expressions of VEGF-A and Ang-2 were increased both at mRNA and protein levels in the diabetic glomeruli. Ang-2 protein was also detected in the urine from diabetic animals but not from the controls. Conversely, the expression of Ang-1 was decreased in diabetic glomeruli. High glucose up-regulated VEGF-A mRNA and Ang-2 mRNA expression and down-regulated Ang-1 mRNA expression in cultured podocyte. VEGF-A alone up-regulated both VCAM-1 and PDGF-B mRNA expression and Ang-1 attenuated the VEGF-A-induced VCAM-1 and PDGF-B mRNA expression in cultured endothelial cell.

Our data indicate that high glucose up-regulate VEGF-A and Ang-2 expressions and down-regulate of Ang-1 expression both *in vivo* and *in vitro*. The altered expression of VEGF-A and angiopoietins in glomeruli could be important for the development of diabetic nephropathy through the modulation of glomerular endothelial gene expression.

Keywords: Diabetic nephropathy, Podocyte, Endothelial cell, VEGF, Angiopoietin,

Diabetic nephropathy, a major microvascular complication of diabetes mellitus, affects approximately one-third of all diabetic patients and is a major cause of morbidity and mortality due to both type 1 and type 2 diabetes ^{1, 2}. This complication has also become the leading cause of end-stage renal disease (ESRD) all over the world. Prevention and treatment of chronic renal insufficiency would require the application of therapies that specifically interfere with the pathogenesis of diabetic nephropathy. However, the molecular mechanisms involved in diabetic nephropathy are largely unknown.

Recently, the involvement of various growth factors and cytokines, including VEGF ³, angiotensin II ⁴, insulin-like growth factor-I ⁵, monocyte chemoattractant protein-1 ⁶ and transforming growth factor- β_1 ⁷, has been reported in the development of diabetic nephropathy.

VEGF is considered a powerful angiogenic and permeability-inducing factor and is known to exert its effects via VEGF receptors ⁸. The levels of VEGF expression seem to be critical: reduction or increase in these levels may lead to glomerulopathy with lesions resembling those seen in preeclampsia or HIV-

associated nephropathy ⁹.

Podocytes also secrete Ang-1, which acts via the endothelial Tie-2 receptor ¹⁰. The Ang-1-Tie-2 signaling loop is essential for vessel maturation and stabilization ¹¹. Ang-1 is also unique in its ability to reduce endothelial cell permeability ¹² and probably modulates some of the effects of VEGF ¹³.

Ang-2 is an antagonistic ligand for Tie-2 in endothelial cells ¹⁴ that inhibits the binding of Ang-1 to Tie-2, and thereby the Ang-1-induced Tie-2 phosphorylation. Endothelial cells of transgenic mice overexpressing Ang-2 have a phenotype similar to those of Ang-1-deficient mice ¹⁵.

In this study, we conducted both in vivo and in vitro experiments to examine the glomerular expression of VEGF-A, Ang-1, and Ang-2 under diabetic conditions and the effects of those growth factors on the glomerular endothelial cell gene expression.

METHODS

Reagents

The following reagents were used in this study: recombinant human VEGF (293-VE; R&D systems, Minneapolis, MN, USA), recombinant human Ang-1 and Ang-2 (2623 and 2923, respectively; Genzyme-Techne, Minneapolis, MN, USA). The following commercially available antibodies were used: goat anti-mouse Ang-2, rabbit anti-human VEGF, and horseradish peroxidase (HRP)-linked donkey anti-goat IgG (sc-7017, sc-507, and sc-2020 from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and HRP-linked goat anti-rabbit IgG (DAKO, Copenhagen, Denmark).

Animals and induction of diabetes

Eight-week-old, male, C57/Bl6J mice were induced diabetes by intraperitoneal injection of streptozotocin (STZ; Sigma Aldrich, St. Louis, MO, USA) freshly dissolved in an ice-cold citrate buffer at a dose of 100 mg/kg once a day for 3 days. All the experiments in this study were performed in accordance with the Guidelines of the Animal Care and Use of Chiba University, Japan.

Mice urine

Mice were placed in individual metabolic cages for 12 h for collection of urine. Urinary albumin concentrations were determined using an enzyme enzyme-linked

immunosorbent assay (ELISA) kit (Albuwell M; Exocell, Philadelphia, PA, USA).

Isolation of glomeruli

Isolation of mice glomeruli was performed as described previously ¹⁶.

Cell culture

Mouse podocytes, conditionally immortalized with a temperature-sensitive variant of the SV40 large T-antigen, were kindly provided by Dr. Peter Mundel (Albert Einstein College of Medicine, NY, USA). Human glomerular endothelial cells (CS-ABI-128, Applied Cell Biology Research Institute, Kirkland WA, USA) were maintained in EBM-2 (CC 3162, CAMBREX Walkersville, MD, USA). Cells at passages 6 to 9 were used for the present experiments.

Immunohistochemistry

Kidneys were frozen in optimal cryotomy temperature (OCT) compound and sectioned into 8- μ m-thick slices. Immunohistochemistry was performed as described previously ¹⁷.

Real-time reverse-transcriptase polymerase chain reaction

The mRNA expression in glomeruli or podocytes was analyzed by real-time polymerase chain reaction (PCR) as described previously ¹⁸. The sense and antisense primers for the gene encoding Ang-1 (Gene Bank Accession No. U83509, 365–432 bp) were 5'-GCAACCAGCGCCGAAAT-3' and 5'-GGCACATTGCCCATGTTGA-3'; those for the gene encoding Ang-2 (Gene Bank Accession No. AF004326, 1213–1284 bp) were 5'-TTCCAGAGGACGTGGAAAGAA-3' and 5'-CTCATTGCCAGCCAGTACTC-3'; those encoding VEGF-A (Gene Bank Accession No. XM192823, 144-210 bp) were 5'-CATCTTCAAGCCGTCCTGTGT-3' and 5'-CTCCAGGGCTTCATCGTTACA-3'; and those encoding Tie-2 (Gene Bank Accession No. X71426, 583-659bp) were 5'-CCCGGCATGAAGTACCTGATAT-3' and 5'-GGCCGAGTACACACCAGCAT-3'. The relative mRNA levels of each molecule were normalized to that of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Gene Bank Accession No. M32599, 757–855bp), which was amplified using the primers, 5'-TGTGTCCGTCGTGGATCTGA-3' and 5'-CCTGCTTCACCACCTTCTTGA-3'.

Western blotting

Western blotting was performed exactly as described in a previous report¹⁹. The medium harvested from podocyte was concentrated to about 4-fold by using the Centricon YM-10 filter (Cat. No 4205, Millipore, Billerica, MA, USA) according to the manufacturer's instructions.

Statistical analysis

The statistical analysis of the results was performed by the unpaired Student t test. P values < 0.05 were considered significant.

RESULTS**Alteration of the glomerular expression of VEGF-A, Ang-1, and Ang-2 under diabetic conditions**

To examine the expression of angiogenesis-related factors during the early stages of the pathogenesis of diabetic glomerulopathy, STZ-induced diabetic mice as well as control mice were divided into 4 groups: mice not injected, those at 2 weeks after injection, those at 4 weeks after injection, and those at 8 weeks after injection. Blood and urine samples were obtained from each group at each time point, and the glomeruli were isolated from renal samples and analyzed for gene and protein expressions. Fasting plasma glucose levels in mice with STZ-induced diabetes were twice those in nondiabetic control mice (Figure 1A). A significant increase was noted in the urinary albumin excretion in diabetic mice at 2 weeks after the STZ injection (Figure 1B). The expression of VEGF-A mRNA in the diabetic mice was significantly higher (by 220%) than that in the non-diabetic control mice (Figure 2A). Ang-2 mRNA expression also increased by 140 and 340% in the diabetic mice from 4 weeks after STZ injection, respectively (Figure 2B). Conversely, mRNA expression level of Ang-1 was significantly lower in the diabetic mice than in non-diabetic control mice at 8 weeks after injection (Figure 2C). There was no significant difference in the glomerular expression level of Tie-2 mRNA between the diabetic and control mice (Figure 2D). Western blot analysis confirmed that glomerular expression of Ang-2 protein in diabetic mice was 1.5-fold that in nondiabetic mice (Figure 2E, right). Ang-2 protein was also detected in the urine samples of diabetic mice, but not in those of control animals

(Figure 2E, left). The increased glomerular expression of VEGF-A protein in diabetic mice was also confirmed by immunohistochemistry, as shown in Figure 2F.

Changes in the expression levels of VEGF-A, Ang-1, and Ang-2 in podocytes cultured under high concentrations of glucose

To understand the molecular mechanisms behind the altered expression of VEGF-A, Ang-1, and Ang-2 under diabetic conditions, we examined the effects of exposure to high glucose levels on VEGF-A, Ang-1, and Ang-2 expressions in a podocyte cell line. Incubation of podocytes under high concentrations of glucose up-regulated VEGF-A mRNA expression by 144% ($P < 0.01$) and Ang-2 mRNA expression by 175% ($P < 0.01$) and down-regulated the expression of Ang-1 mRNA by 82% ($P < 0.01$) (Figures 3A, 3B, and 3C). Western blot analyses also revealed that increased duration of exposure to high glucose concentrations lead to an increase in the expression of Ang-2 protein in a dose-dependent manner (Figure 3D).

The effects of VEGF-A, Ang-1, and Ang-2 on the expressions of PDGF-B and vascular cell adhesion molecule-1 mRNA in cultured endothelial cells

Since the receptors for VEGF-A, Ang-1, and Ang-2 are known to be expressed in endothelial cells, we next examined the effects of VEGF, Ang-1, and Ang-2 on the gene expression in these cells. Cultured human glomerular endothelial cells were treated with recombinant human VEGF-A, Ang-1, or Ang-2 alone or combinations of 2 of these factors, and the mRNA levels of VCAM-1 and PDGF-B were evaluated. VEGF-A up-regulated the mRNA expression of both VCAM-1 and PDGF-B (Figure 4), whereas neither Ang-1 nor Ang-2 alone had any effect on VCAM-1 and PDGF-B mRNA expression (data not shown). Ang-1 attenuated the VEGF-induced over-expression of both VCAM-1 and PDGF-B mRNA. These data are schematically illustrated in Figure 5.

DISCUSSION

In this study, we showed that glomerular expressions of VEGF-A and Ang-2 were increased both at mRNA and protein levels, whereas Ang-1 expressions decreased at the mRNA level in mice with STZ-induced diabetes. Ang-2 protein was also increased in the urine from diabetic animals. High glucose up-regulated VEGF-A mRNA and Ang-2 mRNA expression and down-regulated Ang-1 mRNA expression in cultured podocyte. VEGF-A alone up-regulated both VCAM-1 and

PDGF-B mRNA expression and Ang-1 attenuated the VEGF-A-induced VCAM-1 and PDGF-B mRNA expression in cultured endothelial cell.

Increased renal expression of VEGF-A as well as its receptors, VEGFR-1 and VEGFR-2, has been reported in both type 1 and type 2 diabetes^{20, 21}. Makino and coworkers indicated that the expression of Ang-2 in the kidneys of animals with STZ-induced diabetes increases, while there is no difference in Ang-1 expression^{22, 23}. In their study, the expressions of Ang-1 and Ang-2 in the renal cortex were evaluated at 4 weeks after the STZ injection. Similar to their findings, no change was detected in the expression of Ang-1 at 4 weeks after the STZ injection, but its expression was significantly decreased at 8 weeks after the injection. Therefore, Ang-1 seems to respond to diabetic conditions at a relatively later time point than the other 2 factors.

We would like to emphasize that the gene and protein expressions in this study were examined using isolated glomeruli with 97% purity, rather than the renal cortex, which was used in previous studies. Ang-1 is also known to be expressed in vascular smooth muscle cells in the kidney¹⁰; therefore, our approach would be more direct and accurate in assessing glomerular expressions of genes and proteins.

Notably, we could also detect Ang-2 proteins in the urine samples obtained from diabetic animals, as shown in Figure 2E. On the basis of a previous study that indicated that Ang-2 mRNA and protein are barely detected in healthy, mature glomeruli²⁴, we can infer that urinary Ang-2 levels can be used as an early marker of diabetic nephropathy in the future.

There is increasing evidence indicating the role of podocytes in the development of diabetic nephropathy²⁵. Therefore, we used cultured renal podocytes to examine the effect of high glucose concentrations on the expressions of VEGF-A, Ang-1, and Ang-2 in these cells. Exposure to high glucose concentrations lead to up-regulation of VEGF mRNA and protein expression in podocytes via activation of the α and β II isoforms of protein kinase C (PKC) and extracellular signal-regulated kinases (ERKs)²⁶. However, to our knowledge, this is the first report showing that exposure to high glucose concentrations increases the Ang-2 mRNA expression and decreases Ang-1 mRNA expression in podocytes, although the exact mechanism underlying this alteration in the expression patterns of Ang-1 and Ang-2 remains unclear.

Ang-1 has been reported to act as an anti-permeability and anti-inflammatory agent in vitro ²⁷. Our data indicated that Ang-1 attenuated VEGF-A-mediated overexpression of VCAM-1 and PDGF-B in cultured glomerular endothelial cells (Fig.4). VCAM-1, a cell surface glycoprotein expressed by cytokine-activated endothelium, mediates the adhesion of monocytes and lymphocytes. There is a growing body of evidence implicating inflammatory cells in every step of the pathogenesis of diabetic nephropathy ²⁸. Accumulation of macrophages, increased leukocyte adhesion, and overexpression of chemokines are prominent characteristics of kidney tissues in diabetic patients.

PDGF-B is a potent mitogen as well as a chemoattractant for fibroblasts, osteoblasts, smooth muscle cells, and mesangial cells ²⁹. A recent study has shown that the abnormal levels of glomerular expression of PDGF-B protein in mice leads to the glomerular abnormalities similar to those seen in diabetes, including glomerulosclerosis and proteinuria ³⁰. Therefore, Ang-1 expressed in glomeruli seems to act as anti-inflammatory and anti-sclerotic agent directly and/or through the attenuation of VEGF-A-mediated up-regulation of VCAM-1 and PDGF-B expression in the glomerular endothelial cells.

Ang-2 is known to antagonize the Ang-1-mediated Tie-2 signaling. Ang-2 binds to Tie-2 without leading to signal transduction in Tie-2-expressing endothelial cells ¹⁴. The expressions of Ang-1 and Ang-2 should be tightly regulated to maintain glomerular homeostasis. Recently, it has been reported that the over-expression of Ang-2 in podocyte lead to proteinuria and apoptosis in glomerular endothelia ³¹. Our data indicated that diabetic conditions, specifically exposure to high glucose concentrations, induce an imbalance in the expression levels of Ang-1 and Ang-2, which causes enhanced VEGF signaling leading to over-expression of VCAM-1 and PDGF-B in the glomerular endothelial cells.

Interestingly, our data showed that Ang-2 alone had no effect on VCAM-1 expression, but tended to increase VCAM-1 expression in the presence of VEGF-A. A recent report indicated that Ang-2 is capable of sensitizing endothelial cells toward TNF- α and modulating TNF- α -induced expressions of intercellular adhesion molecule 1 and VCAM-1 ³². Therefore, it can be inferred that Ang-2 might sensitize the endothelial cells to VEGF-A, thereby modulating the expression of endothelial cell adhesion molecules.

In conclusion, our data suggested that altered expressions of VEGF-A and

angiopoietins in diabetes could be important factors in the development of glomerular changes in diabetes and might represent a potential therapeutic target for the prevention of diabetic nephropathy. However, further studies are necessary to confirm these hypotheses.

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CONFLICT OF INTEREST

None

FIGURE LEDENDS

Figure 1

Figure 1. The level of blood glucose and urinary albumin excretion in mice with streptozotocin (STZ)-induced diabetes.

(A) The blood glucose levels in the mice with STZ-induced diabetes and controls were compared at each time point.

Data are expressed as means $\pm$ SEM (n = 3). * P < 0.01 vs. controls.

(B) The mice were maintained in individual metabolic cages for 12 h for urine collection. The urine volume was measured gravimetrically, and urinary albumin concentrations were determined by performing an enzyme-linked immunosorbent assay. The measurements obtained at each time point were compared between mice with STZ-induced diabetes and control mice.

Data are expressed as means $\pm$ SEM (n = 3). * P < 0.01 vs. controls.

Figure 2

Figure 2. Alterations in the glomerular expressions of VEGF-A, Ang-1, Ang-2, and Tie-2 mRNA in mice with streptozotocin (STZ)-induced diabetes. The glomeruli were isolated both from diabetic and control mice at each time point, and the expression levels of VEGF-A (A), Ang-2 (B), Ang-1 (C), and Tie-2 (D) mRNA were measured by quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR).

Data are expressed as means $\pm$ SEM ($n = 3$). * $P < 0.05$ vs. controls.

(E) The glomerular lysates (left panel) as well as urine samples (right panel) were subjected to western blotting to evaluate protein expression of Ang-2. The bands were then quantified by densitometry. The glomerular Ang-2 protein expression patterns shown in this figure (left panel) are representative of 3 independent experiments providing essentially similar results. The bars in the right panel represent mean $\pm$ SEM ($n = 4$). * $P < 0.05$ vs. controls.

(F) The expression of VEGF-A protein in kidney was compared between diabetic and control mice by immunohistochemical analysis. Arrows indicate glomeruli.

Figure 3

Figure 3. The effects of high glucose concentrations on the expressions of VEGF, Ang-1 and Ang-2 in cultured podocytes. Podocytes were cultured for 24 h in a medium containing glucose at normal (5.5 mM) or high (25 mM) concentration. The total RNA was then extracted from the cells, and the expression levels of VEGF (A), Ang-2 (B), and Ang-1 (C) mRNA were evaluated by quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR).

Data are expressed as means $\pm$ SEM ($n = 3$). * $P < 0.01$ vs. controls.

(D) The medium used for harvesting the podocytes was concentrated and subjected to western blotting to evaluate protein expression of Ang-2. The bands were then quantified by densitometry. The bands shown in this figure are representative of 3 independent experiments providing essentially similar results.

Figure 4

Figure 4. The effects of VEGF-A, Ang-1, and Ang-2 on the expressions of PDGF-B and VCAM-1 mRNA in cultured endothelial cells. Cultured human glomerular endothelial cells were treated with recombinant human VEGF-A, Ang-1, or Ang-2 alone or combinations of 2 of these factors, and the mRNA levels of VCAM-1 and PDGF-B were evaluated by quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Data are expressed as means $\pm$ SEM (n = 3). * P < 0.01 vs. controls. ** P < 0.05 vs. controls.

Figure 5

Figure 5. Schematic illustration of the alteration in the gene expression of both podocytes and glomerular endothelial cells induced by exposure to high glucose concentrations.

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