Antiangiogenic Endostatin Peptide Ameliorates Renal Alterations in the Early Stage of a Type 1 Diabetic Nephropathy Model

Kunihiro Ichinose,1 Yohei Maeshima,1 Yoshihiko Yamamoto,1 Hiroyuki Kitayama,1 Yuki Takazawa,1 Kumiko Hirokoshi,1 Hitoshi Sugiyama,1 Yasushi Tamasaki,1 Katsumi Eguchi,2 and Hirofumi Makino1

Diabetic nephropathy is one of the major microvascular complications in diabetes and is the leading cause of end-stage renal disease worldwide. Among various factors, angiogenesis-associated factors such as vascular endothelial growth factor (VEGF)-A and angiopoietin (Ang)-2 are involved in the development of diabetic nephropathy. We previously reported the therapeutic efficacy of antiangiogenic tumstatin peptide in the early diabetic nephropathy mouse model. Here, we examine the effect of endostatin peptide, a potent inhibitor of angiogenesis derived from type XVIII collagen, in preventing progression in the type 1 diabetic nephropathy mouse model. Endostatin peptide did not affect hyperglycemia induced by streptozotocin (STZ). Glomerular hypertrophy, hyperfiltration, and albuminuria were significantly suppressed by endostatin peptide (5 mg/kg) in STZ-induced diabetic mice. Glomerular mesangial matrix expansion, the increase of glomerular type IV collagen, endothelial area (CD31+), and F4/80+ monocyte/macrophage accumulation were significantly inhibited by endostatin peptide. Increase in the renal expression of VEGF-A, flk-1, Ang-2, an antagonist of angiopoietin-1, transforming growth factor-β1, interleukin-6, and monocyte chemoattractant protein-1 was inhibited by endostatin peptide in diabetic mice. Decrease of nephrin mRNA and protein in diabetic mice was suppressed by treatment with endostatin peptide. The level of endostatin in the renal cortex and sera was increased in diabetic mice. Endogenous renal level of endostatin were decreased in endostatin peptide–treated groups in parallel with VEGF-A. Although serum levels of endostatin were decreased in the low-dose endostatin-peptide group, high-dose administration resulted in elevated serum levels of endostatin. These results demonstrate the potential use of antiangiogenic endostatin peptide as a novel therapeutic agent in diabetic nephropathy. Diabetes 54:2891–2903, 2005

From the 1Department of Medicine and Clinical Science, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan; and the 2First Department of Internal Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan.

Address correspondence and reprint requests to Dr. Yohei Maeshima, Department of Medicine and Clinical Science, Okayama University Graduate School of Medicine and Dentistry, 2–5–1 Shikata-cho, Okayama, 700-8558, Japan. E-mail: ymaeshim@md.okayama-u.ac.jp.

Received for publication 4 March 2005 and accepted in revised form 11 July 2005.

Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org.

Ang, angiopoietin; Ccr, creatinine clearance; ELISA, enzyme-linked immunosorbent assay; ESRD, end-stage renal disease; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; NC1, noncollagenous domain 1; STZ, streptozotocin; TGF, transforming growth factor; UACR, urinary albumin-to-creatinine ratio; VEGF, vascular endothelial growth factor.

© 2005 by the American Diabetes Association.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Diabetic nephropathy is a complication in 30–40% of patients with type 2 diabetes and is the most common pathological disorder predisposing end-stage renal disease (ESRD) in Japan and in the Western world. Early alterations in diabetic nephropathy include glomerular hyperfiltration, glomerular and tubular epithelial hypertrophy, and the development of microalbuminuria, followed by the development of glomerular basement membrane thickening, the accumulation of mesangial matrix, and overt proteinuria, eventually leading to glomerulosclerosis and ESRD (1). The involvement of various growth factors and cytokines including angiotensin II, IGF-I, monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6), and transforming growth factor (TGF)-β1 in the development of diabetic nephropathy have been reported (2).

Angiogenesis, the development of new blood vessels from preexisting ones, is involved in physiological events such as wound repair, but uncontrolled neovascularization is associated with a number of pathological disorders, including tumor growth, rheumatoid arthritis, and diabetic retinopathy (3). Vascular endothelial growth factor (VEGF)-A, a potent stimulator of angiogenesis, promotes endothelial cell proliferation, migration, and tube formation (4). VEGF-A also induces vascular permeability (5) in association with inflammation and other pathological circumstances. The protein and mRNA level of VEGF-A and the receptor of VEGF-A, flk-1/kinase domain region domain region were reported to be upregulated in the early and advanced stages of experimental diabetic nephropathy (6,7). Recent animal studies using neutralizing anti–VEGF-A antibody further demonstrated the involvement of VEGF-A in early glomerular hypertrophy and mesangial matrix accumulation in the progressive stage of diabetic nephropathy (8,9).

Angiopoietin (Ang)-1, a major physiological ligand for endothelial receptor Tie2, is responsible for the recruitment and stable attachment of pericytes resulting in the maturation of newly formed blood vessels (10). Ang-2 competitively inhibits the binding of Ang-1 to Tie2, acting like a natural antagonist, and renders blood vessels “unstable” (11). Ang-2 loosens the attachment of pericytes and synergizes with VEGF-A to stimulate angiogenesis (11,12). However, when insufficient angiogenic stimuli are present, Ang-2 causes endothelial cell apoptosis and vessel regression (11). During kidney development, Ang-1, Ang-2, and Tie2 are highly expressed and play pivotal roles in the maturation of glomeruli and renal blood vessels (13). We
previously reported the upregulation of Ang-2 in the type 1 mouse diabetic nephropathy model, although the expression of Ang-1 was not altered (14).

Nephrin, a recently found podocyte protein, is crucial for maintaining the integrity of the interpodocyte slit membrane structure and maintenance of an intact filtration barrier. In diabetic nephropathy, the protein level of nephrin decreases possibly via the loss of nephrin into urine due to synthesis of the splice variant isoform of the nephrin lacking a transmembrane domain (15,16).

A previous study demonstrated that the increased glomerular filtration surface in diabetic nephropathy results from the formation of new glomerular capillaries in accordance with a slight elongation of the preexisting capillaries (17), analogous to the changes observed in pathological diabetic retinopathy in the development of diabetic nephropathy similar to diabetic retinopathy.

Endostatin, a 20-kDa COOH-terminal noncollagenous domain 1 (NCI) domain of type XVIII collagen, possesses potent antiangiogenic activity (18). Endostatin inhibits endothelial cell proliferation, migration, and tube formation in vitro (19) and possesses a potent inhibitory effect on tumor growth in vivo (18). The inhibitory effects of endostatin on the expression of VEGF-A in tumor cells and on vascular permeability have been reported (20). A recent report describes the antiangiogenic activity of synthetic peptide derived from the NH2-terminal domain of endostatin (5). Endostatin on the expression of VEGF-A in tumor cells and on vascular permeability have been reported (20). A recent report describes the antiangiogenic activity of synthetic peptide derived from the NH2-terminal domain of endostatin in vitro and in vivo (21,22). Several recent reports indicate possible cell-surface receptors for the antiangiogenic activity of endostatin, i.e., α5β1-integrin or glypicans (23,24).

In the present study, we demonstrate the therapeutic efficacy of endostatin peptide in ameliorating alterations in the streptozotocin (STZ)-induced mouse type 1 diabetic nephropathy model. Treatment with endostatin peptide markedly suppressed glomerular hypertrophy, hyperfiltration, and urinary albumin excretion as well as the expansion of mesangial matrix and the accumulation of monocytes/macrophages. These effects are thought to be mediated through the downregulation of proangiogenic signals, VEGF-A/flk-1 and Ang-2; the suppression of TGF-β1, IL-6, and MCP-1; and the recovery of nephrin.

### RESEARCH DESIGN AND METHODS

**Induction of diabetes and experimental protocols.** The experimental protocol was approved by the Animal Ethics Review Committee of Okayama University Graduate School of Medicine and Dentistry. Female C57BL6 mice were fed a standard pellet laboratory diet and were provided with water ad libitum. Type 1 diabetes was induced by low-dose STZ injection as detailed by the National Institute of Diabetes and Digestive and Kidney Diseases Consor- tium for Animal Models of Diabetic Complications’ protocol (available from http://www.amdc.org) with modification. Weight-matched 7- to 8-week-old mice received three intraperitoneal injections of STZ (Sigma, St. Louis, MO; 100 mg/kg body wt) dissolved in 10 mmol/L sodium citrate, pH 5.5. Control mice received injections with buffer alone. STZ or citrate buffer was administered at three time points occurring at 48-h intervals during the 1st week. Six days after the third injection of STZ, mice with blood glucose in the range of 13.9–22.2 mmol/l were divided into three subgroups: 1) endostatin peptide treatment (1 mg/kg body wt), 2) endostatin peptide treatment (5 mg/kg body wt), and 3) vehicle buffer treatment, extending for 4 weeks (n = 6 for each subgroup). Then, mice of groups 1 and 2 received continuous infusion of endostatin peptide, and mice of group 3 received vehicle buffer (PBS) via subcutaneously implanted osmotic minipumps (Alzet model 2004; Alza, Palo Alto, CA).

Blood glucose and urine samples were monitored every week, and when needed, diabetic mice were given supportive insulin treatment (Ultratard; Novo Nordisk; 1 IU/kg body wt twice a week) to maintain blood glucose in the range of 13.9–22.2 mmol/l and to prevent ketosis. No mice died, and no signs of apparent exhaustion were observed during the experimental period. Two, 3, and 4 weeks after the implantation of osmotic minipumps, individual 24-h urine sample collections were performed using metabolic cages, and the body weight was measured. Nonfasting blood samples were drawn from the retro-orbital venous plexus using heparinized capillary tubes under anesthesia at the time of killing. Kidney weight was measured just after killing. Endostatin peptide (amino acid sequence, H-HSHRDFQPVLHLVALNSPLSGGMRGIR-OH) encompassing 1-27 amino acid portion of human endostatin, was synthesized and purified by high-performance liquid chromatography (Kurabo, Osaka, Japan; Multiple Peptide Systems, San Diego, CA) and characterized as previously described (25). Endostatin peptide with a purity >97% was used and dissolved in PBS to be used for the animal experiments. The dosage of endostatin peptide used in the present study was determined according to a previous study using endostatin peptide (5 mg/kg) and studies using recombinant endostatin (0.3–10 mg/kg) in animal models (18,21). The dosage of recombinant human endostatin (30–300 mg·m·day”) in human clinical trials in patients with advanced solid tumors failed to exhibit any toxic side effects (26), suggesting the feasibility of the dosage used in the present study in humans.

Blood glucose was measured in tail-vein blood, and urine was tested for ketone bodies and glucose by the SRL (Okayama, Japan). Serum and urinary creatinine levels were measured by the enzymatic colorimetric method as described previously (14). Urinary albumin concentration was measured by nephelometry (Organon Teknika-Cappel, Durham, NC) using anti-mouse albumin antibody (ICN Pharmaceuticals, Aurora, OH) as previously described (14). Results were normalized to the urinary creatinine levels and expressed as urinary albumin-to-creatinine ratio (UACR). The creatinine clearance (CCr) was calculated and expressed as milliliters per minute per 100 g body wt.

**Histological analysis and immunohistochemistry.** At 4 weeks after start- ing treatment, kidneys were removed, fixed in 10% buffered formalin, and embedded in paraffin. Sections (3-μm) were stained with periodic acid-Schiff for light microscopic observation (online appendix [available at http://diabetes. diabetesjournals.org]). Immunohistochemistry was performed using frozen sections as previously described (14,27) (online appendix).

**RNA extraction, quantitative real-time PCR, and immunoblot assay.** RNA extraction and real-time PCR were performed as previously described with modifications (14) (online appendix). The immunoblot assay was performed as previously described (14,28) (online appendix).

**Statistical analysis.** All values are expressed as means ± SE. A Kruskal-Wallis test with post hoc comparisons using the Scheffe’s test were used for inter-group comparisons of multiple variables. Statistical analysis was performed by StatView software (Abacus Concepts, Berkeley, CA). A level of P < 0.05 was considered statistically significant.

### RESULTS

**Changes in blood glucose, body weight, kidney weight, kidney weight/body weight, and blood pressure.** Administration of endostatin peptide at different dosages did not alter plasma glucose concentrations compared with diabetic mice treated with vehicle buffer after 4 weeks of treatment (Table 1). Body weight was significantly lower in all of the diabetic groups compared with nondiabetic animals. There was no significant difference in body weight between diabetic mice treated with endostatin peptide and diabetic animals treated with vehicle buffer. Kidney weight was significantly lower in endostatin-treated groups compared with vehicle-treated diabetic animals. Diabetic animals exhibited significantly greater kidney

<table>
<thead>
<tr>
<th>Group</th>
<th>Body wt (g)</th>
<th>Kidney wt (mg)</th>
<th>Blood glucose (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nondiabetic</td>
<td>26.3 ± 0.6</td>
<td>288.7 ± 11.1</td>
<td>7.3 ± 0.4</td>
</tr>
<tr>
<td>Diabetic (vehicle)</td>
<td>21.5 ± 1.0*</td>
<td>293.2 ± 4.8</td>
<td>25.4 ± 3.1*</td>
</tr>
<tr>
<td>Diabetic (ES, 1 mg/kg)</td>
<td>22.1 ± 0.6*</td>
<td>269.4 ± 20.7</td>
<td>25.0 ± 1.8*</td>
</tr>
<tr>
<td>Diabetic (ES, 5 mg/kg)</td>
<td>21.4 ± 1.1*</td>
<td>228.7 ± 6.5*††</td>
<td>25.6 ± 2.5*††</td>
</tr>
</tbody>
</table>

Data are means ± SE, n = 5 in each group. Vehicle was buffer treated. ES, endostatin peptide treated. *P < 0.05 vs. nondiabetic animals. †P < 0.01 vs. vehicle. ‡P < 0.05 vs. mice treated with endostatin peptide (1 mg/kg).

**Histological analysis and immunohistochemistry.** At 4 weeks after start- ing treatment, kidneys were removed, fixed in 10% buffered formalin, and embedded in paraffin. Sections (3-μm) were stained with periodic acid-Schiff for light microscopic observation (online appendix [available at http://diabetes. diabetesjournals.org]). Immunohistochemistry was performed using frozen sections as previously described (14,27) (online appendix).

**RNA extraction, quantitative real-time PCR, and immunoblot assay.** RNA extraction and real-time PCR were performed as previously described with modifications (14) (online appendix). The immunoblot assay was performed as previously described (14,28) (online appendix).

**Statistical analysis.** All values are expressed as means ± SE. A Kruskal-Wallis test with post hoc comparisons using the Scheffe’s test were used for inter-group comparisons of multiple variables. Statistical analysis was performed by StatView software (Abacus Concepts, Berkeley, CA). A level of P < 0.05 was considered statistically significant.

**RESULTS**
weight-to-body weight ratio compared with nondiabetic mice (Fig. 1). Treatment with endostatin peptide resulted in decreased kidney weight-to-body weight ratio compared with vehicle-treated diabetic mice in a dose-dependent manner (Fig. 1). There was no significant difference in blood pressure among nondiabetic animals, diabetic mice treated with endostatin peptide, and diabetic animals treated with vehicle buffer (Table 2). Also, there was no difference in blood pressure between nondiabetic mice treated with endostatin peptide (5 mg/kg) and nondiabetic animals (data not shown).

**Changes in serum creatinine, CCr, and urinary albumin excretion.** Serum creatinine levels did not significantly differ among the experimental groups (nondiabetic control, 0.140 ± 0.014 mg/dl; diabetic mice treated with vehicle buffer, 0.133 ± 0.011 mg/dl; diabetic mice treated with endostatin peptide at 1 mg/kg, 0.140 ± 0.011 mg/dl; and diabetic mice treated with endostatin peptide at 5 mg/kg, 0.133 ± 0.012 mg/dl). To evaluate the effect of endostatin peptide on preventing hyperfiltration induced by STZ, we measured CCr and urinary albumin excretion (Fig. 2). Although diabetic mice treated with vehicle buffer showed a marked elevation of CCr and UACR, endostatin peptide treatment resulted in the suppression of STZ-induced increase of CCr (nondiabetic mice, 0.59 ± 0.14 ml·min⁻¹·100 g body wt⁻¹; diabetic mice treated with vehicle buffer, 1.13 ± 0.12 ml·min⁻¹·100 g body wt⁻¹; diabetic mice treated with endostatin peptide at 1 mg/kg, 0.78 ± 0.07 ml·min⁻¹·100 g body wt⁻¹; and diabetic mice treated with endostatin peptide at 5 mg/kg, 0.59 ± 0.14 ml·min⁻¹·100 g body wt⁻¹) and UACR (nondiabetic mice, 27.0 ± 3.1 µg albumin/mg creatinine; diabetic mice treated with vehicle buffer, 109.3 ± 24.6 µg albumin/mg creatinine; diabetic mice treated with endostatin peptide at 1 mg/kg, 69.4 ± 16.8 µg albumin/mg creatinine; and diabetic mice treated with endostatin peptide at 5 mg/kg, 45.2 ± 6.3 µg albumin/mg creatinine, at 4 weeks after starting treatment) in a dose-dependent manner. The increase of UACR in diabetic mice was suppressed by a higher dosage of endostatin peptide at 2, 3, and 4 weeks after the initiation of treatment, and administration of endostatin peptide at a lower dosage resulted in the suppression of UACR at 3 and 4 weeks. The development of glomerular hyperfiltration in the diabetic mice was significantly prevented by treatment with endostatin peptide at 5 mg/kg.

**Histology and morphometric analysis.** Histological examination of the kidneys revealed glomerular hypertrophy and expansion of the mesangial area induced by STZ (Fig. 3A–D). After 4 weeks of treatment by endostatin peptide, glomerular hypertrophy and mesangial matrix expansion induced by STZ were significantly inhibited compared with vehicle buffer treatment (Fig. 3E and F). Systemic administration of endostatin peptide failed to exhibit any pathological alterations in liver or heart of diabetic mice or of nondiabetic mice (data not shown).

**Immunohistochemical analysis of glomerular type IV collagen expression.** To further evaluate the therapeutic effect of endostatin peptide in the diabetic nephropathy model, the expression level of type IV collagen was examined by immunofluorescence staining (Fig. 4A–E). The amount of type IV collagen in glomeruli was increased in the diabetic group (Fig. 4B) compared with nondiabetic mice (Fig. 4A). Enhanced immunoreactivity in diabetic mice was observed mainly in the glomerular basement membrane and mesangial area. Treatment with endostatin peptide dose dependently decreased the accumulation of type IV collagen induced by STZ (Fig. 4C and D). Quantitative analysis revealed a significant inhibitory effect of endostatin peptide on glomerular accumulation of type IV collagen induced by STZ compared with vehicle buffer treatment (Fig. 4E).

**Expression of TGF-β1 mRNA.** TGF-β1 serves as a profibrotic growth factor involved in the expansion of mesangial matrix and renal hypertrophy in diabetic nephropathy (29). Diabetic mice exhibited increased expression of TGF-β1 mRNA compared with nondiabetic animals in the renal cortex as assessed by real-time PCR. Treatment with endostatin peptide resulted in the suppression of TGF-β1 mRNA expression in a dose-dependent manner (Fig. 4F). These results suggest that endostatin peptide suppresses the increase of glomerular type IV collagen, mesangial matrix expansion, and renal hypertrophy via, at least in part, downregulating TGF-β1.

**Immunohistochemical analysis.** We next examined the expression of endothelial cell marker CD31 in glomeruli. In nondiabetic mice, CD31 was detected in glomerular capillaries (Fig. 5A), and increased expression of CD31 in glomerular capillary endothelial cells was observed in control diabetic mice (Fig. 5B). Treatment with endostatin peptide at 5 mg/kg markedly suppressed the increase of the CD31⁺ area in glomeruli (Fig. 5D). Quantitative analysis revealed that the STZ-induced increase in the glomerular capillary area was significantly suppressed by endostatin peptide at a higher dosage (Fig. 5F). These

**TABLE 2**

<table>
<thead>
<tr>
<th>Group</th>
<th>SBP (mmHg)</th>
<th>DBP (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nondiabetic</td>
<td>109.3 ± 1.6</td>
<td>66.6 ± 1.9</td>
</tr>
<tr>
<td>Diabetic (vehicle)</td>
<td>108.0 ± 2.7</td>
<td>63.6 ± 2.1</td>
</tr>
<tr>
<td>Diabetic (ES, 1 mg/kg)</td>
<td>111.0 ± 2.5</td>
<td>64.9 ± 3.7</td>
</tr>
<tr>
<td>Diabetic (ES, 5 mg/kg)</td>
<td>111.3 ± 1.7</td>
<td>65.6 ± 2.6</td>
</tr>
</tbody>
</table>

Data are means ± SE, n = 5 in each group. Vehicle was buffer treated. DBP, diastolic blood pressure; ES, endostatin peptide treated; SBP, systolic blood pressure.
results demonstrate that treatment with endostatin peptide resulted in the inhibition of increase in the glomerular CD31+ endothelial area induced by hyperglycemia possibly via its potent antiangiogenic efficacy.

We then examined the expression of mouse monocyte/macrophage surface marker F4/80 in glomeruli. In diabetic mice, the number of F4/80+ cells was significantly increased compared with the nondiabetic control (Fig. 6A–E). Treatment with endostatin peptide markedly decreased the accumulation of monocyte/macrophage in glomeruli in a dose-dependent manner, suggesting the anti-inflammatory action of endostatin peptide in this model.

Expression of IL-6, MCP-1 mRNA, VEGF-A, and receptor flk-1. IL-6 and MCP-1 had been previously reported as critical cytokines involved in leukocyte infiltration in association with the development of inflammatory lesions. The expression of IL-6 and MCP-1 mRNA was assessed by real-time PCR. Diabetic mice exhibited increased expression of IL-6 and MCP-1 mRNA compared with nondiabetic control mice. Treatment with endostatin peptide resulted in significant suppression of IL-6 and MCP-1 mRNA expression (Fig. 6F and G). The effect of endostatin peptide on the expression of proangiogenic factor VEGF-A and corresponding receptors flk-1 in the renal cortex was studied by immunoblot assay. The level of VEGF-A and flk-1 was significantly increased in diabetic mice, which is consistent with previous reports (6,14). Treatment with endostatin peptide significantly suppressed the STZ-induced increase of VEGF-A and flk-1 in a dose-dependent manner (Fig. 7A–D).

Immunohistochemical analysis of VEGF-A. We next examined the expression of mouse VEGF-A in glomeruli by indirect immunohistochemistry. In diabetic mice, immunoreactivity for VEGF-A was increased compared with nondiabetic control, mainly localized to podocytes (Fig. 7E–H). Treatment with endostatin peptide resulted in decreased immunoreactivity for VEGF-A in glomeruli compared with vehicle-treated diabetic mice.

VEGF-A enzyme-linked immunosorbent assay. Circulating VEGF-A levels were next determined by enzyme-linked immunosorbent assay (ELISA) as described in RESEARCH DESIGN AND METHODS (and the online appendix). The serum levels of VEGF-A were significantly increased in diabetic mice compared with nondiabetic control mice (Fig. 7I). Treatment with endostatin peptide resulted in markedly suppressed serum levels of VEGF-A compared with vehicle-treated diabetic mice.

Expression of Ang-1, Ang-2, receptor Tie2, nephrin mRNA, and protein. The effect of endostatin peptide on the expression of angiogenesis-associated factors Ang-1 and Ang-2 and corresponding receptors Tie2 in the renal cortex was studied by immunoblot assay. The expression of Ang-1 was detected in undiseased mice and not significantly altered by STZ. Treatment with endostatin peptide did not affect the expression of Ang-1 (Fig. 8A and B). In contrast, the protein level of Ang-2 was increased in diabetic mice compared with undiseased mice, and treatment with endostatin peptide resulted in significant dose-dependent reduction of Ang-2 in diabetic mice compared with vehicle-treated diabetic mice (Fig. 8C and D). The level of Tie2 was mildly decreased in diabetic mice compared with nondiabetic control mice, and treatment with endostatin peptide did not affect the level of Tie2 in diabetic mice (Fig. 8E and F). These experiments were repeated three times with similar results. The expression of nephrin, an essential component of the slit-diaphragm involved in the regulation of the glomerular filtration barrier, was studied using real-time PCR and immunoblot assay. Vehicle-treated diabetic mice exhibited reduced expression levels of nephrin mRNA and protein compared with the nondiabetic control group. Treatment with endostatin peptide resulted in the recovery of nephrin mRNA and protein expression in a dose-dependent manner (Fig. 9A–C). These results suggest the supportive role of endostatin peptide in maintaining nephrin and thus the glomerular filtration barrier possibly associated with the suppression of albuminuria induced by STZ.

Expression of α5β1-integrin in glomeruli. We next studied the localization of α5β1-integrin, a cell surface receptor for endostatin, in diabetic glomeruli. Although the expression of α5-integrin was faintly observed in nondiabetic glomeruli, vehicle-treated diabetic mice revealed enhanced expression of α5-integrin in glomeruli (Fig. 10A). Double immunofluorescent staining exhibited that α5-integrin in glomeruli in diabetic mice was mostly localized to glomerular endothelial cells expressing CD31, suggesting the primary glomerular cells responsible for the action of endostatin peptide to be glomerular endothelial cells in the present diabetic nephropathy model.
Renal expression and serum levels of endostatin. The expression of endogenous endostatin in the renal cortex in experimental groups was studied by immunoblot assay. The level of endostatin was significantly increased in vehicle-treated diabetic mice compared with nondiabetic control mice. Treatment with endostatin peptide significantly suppressed the STZ-induced increase of endogenous endostatin in a dose-dependent manner (Fig. 10B and C). Serum levels of endostatin were similarly increased in vehicle-treated diabetic mice compared with nondiabetic control mice (Fig. 10D). Interestingly, diabetic mice treated with high-dose endostatin peptide exhibited increased serum levels of endostatin compared with diabetic mice treated with low-dose endostatin peptide, suggesting possible detection of endostatin peptide in sera by the present ELISA system. In support of this speculation, serum levels of endostatin in nondiabetic mice treated with endostatin peptide (5 mg/kg) showed significant elevation compared with untreated nondiabetic mice (data not shown), again suggesting possible detection of exogenously administered endostatin peptide.
of mesenchymal cells to endothelial tubes and differentiation to “pericytes,” resulting in mature, “nonleaky” blood vessels (10). Ang-2 is the natural antagonist of Ang-1 and loosens the attachment of pericytes, resulting in the promotion of sprouting angiogenesis in the presence of VEGF-A (11). The involvement of VEGF-A in progressing diabetic nephropathy has been demonstrated in a number of previous reports, suggesting the therapeutic potential of the inhibition of VEGF-A signaling (6–9). A recent report describes that the degree of neovascularization was significantly increased in patients with diabetic nephropathy and correlates with the expression of VEGF-A mRNA (30). The involvement of angiopeptins has been intensively demonstrated in the setting of diabetic retinopathy; the increased expression of VEGF-A (31) and the therapeutic efficacy of Ang-1 associated with anti-inflammatory bioactivities have been reported in diabetic retinopathy (32). The morphological phenomenon observed in diabetic nephropathy (capillary elongation and increased glomerular capillary number) suggests the involvement of the angiogenic process in analogy with diabetic retinopathy, thus implicating the possible involvement of Ang-1 and Ang-2 in the progression of diabetic nephropathy in addition to VEGF-A.

Type XVIII collagen is a major proteoglycan in basement membranes composed of an NH₂-terminal noncollagenous domain, multiple interrupted triple-helical regions, and COOH-terminal NC1. Endostatin is the 20-kDa fragment of NC1 domain of type XVIII collagen and possesses a potent antiangiogenic activity (18). Endostatin inhibits proliferation, migration, and tube formation and induces cell-cycle arrest of endothelial cells in vitro (18). Endostatin also serves as an inhibitor of tumor and angiogenesis by suppressing the endogenous expression of VEGF-A (20). Endostatin also blocks VEGF-A–mediated proangiogenic signaling via direct interaction with flk-1 (33). Recent reports describe the therapeutic potential of endostatin in non-neoplastic disorders with the involvement of the angiogenic process such as rheumatoid arthritis and proliferative diabetic retinopathy (34,35).

In the present study, we used an STZ-induced type 1 diabetes mouse model to demonstrate the therapeutic efficacy of endostatin peptide in early diabetic nephropathy. Although renal failure is not reproduced well in animal models of diabetic nephropathy, some of the characteristic early alterations in human diabetic nephropathy such as increased albuminuria, the development of glomerular hyperfiltration, and some of the characteristic histopathological changes can be observed in the present diabetic mouse model (36). Continuous administration of endostatin peptide did not affect hyperglycemia or body weight loss induced by STZ, similar to our recent observation using tumstatin peptide, endogenous angiogenesis inhibitor derived from type IV collagen (14). Although diabetic mice exhibited significant weight loss, the extent of reduced body weight was comparable with previous reports using this model, and these mice failed to show any signs of apparent exhaustion during the experimental period. In addition, treatment with endostatin peptide did not give rise to any pathological alterations in heart or liver and failed to affect wound repair, which is consistent with a previous report (37). In vehicle buffer–treated diabetic mice, characteristic changes in early diabetic nephropathy such as increased urinary albumin excretion, glomerular hypertrophy, glomerular hyperfiltration as evidenced by increased Ccr, and increase in kidney weight...
The pivotal role of TGF-β in the development of diabetic nephropathy, especially in the accumulation of extracellular matrix in glomeruli, has been well characterized (38). In the present study, increase in the level of renal TGF-β1 mRNA was significantly suppressed by endostatin peptide, potentially associated with therapeutic efficacy on the accumulation of extracellular matrix in diabetic nephropathy. Recent reports describe that connective tissue growth factor mediated the effect of TGF-β in the process of mesangial matrix expansion and mesangial cell hypertrophy in diabetic nephropathy (39). The role of VEGF-A/flk-1 in inducing the expression of connective tissue growth factor in retinal capillary cells in association with the development of retinal neovascular diseases has been reported (40).

Recently, Chen et al. (41) demonstrated that podocyte-derived VEGF-A induced by TGF-β1 stimulated the production of α3(IV) collagen, a component of α-chains in glomerular basement membrane. Therefore, the inhibitory effect of endostatin peptide on the increase of glomerular type IV collagen and mesangial matrix expansion may, at least in part, be mediated via downregulation of VEGF-A/flk-1, which presumably is involved in mediating the profibrotic effect of TGF-β1 in diabetic nephropathy.

Treatment with endostatin peptide suppressed the increase of the CD31+ glomerular endothelial area in diabetic mice. This effect may be mediated via potent antiangiogenic efficacy of endostatin peptide. Increased accumulation of monocytes/macrophages in glomeruli has been reported in diabetic nephropathy (14). Considering the effect of VEGF-A in promoting vascular permeability, it is possible that increased monocyte/macrophage accumulation in diabetic nephropathy may be partially mediated via stimulation of VEGF-A. Because endostatin potently inhibits the VEGF-A-induced angiogenic action in the setting of tumor (20) and diabetic retinopathy (35), we speculate that the inhibitory effect of endostatin peptide on the recruitment of F4/80+ monocytes/macrophages was mediated, at least in part, via interference in the VEGF-A signal, thus leading to decreased vascular permeability. In addition, treatment with endostatin peptide resulted in the suppression of IL-6 and MCP-1 expression in diabetic animals. The inhibitory effect of endostatin peptide on
these cytokines (chemokines) may mediate its therapeutic role on monocyte/macrophage infiltration.

We next examined the effect of endostatin peptide on the level of angiogenesis-associated factors. The level of VEGF-A and flk-1 was markedly increased in the renal cortex of diabetic mice, consistent with previous studies (6,7,14). Treatment with endostatin peptide significantly suppressed the increase of VEGF-A as well as flk-1 in the kidneys of diabetic mice. The therapeutic effect of endostatin peptide in early diabetic nephropathy, at least in part, may be attributed to the inhibition of the VEGF-A pathway in analogy with previous studies using neutralizing anti–VEGF-A antibodies (8,9). Strategies using endostatin peptide offer several advantages over anti–VEGF-A antibodies such as 1) the lack of immune response due to the origin of endostatin peptide (endogenous human type XVIII collagen), and 2) feasibility in the production of synthetic peptide with inexpensive cost.

We observed a significant inhibitory effect of endostatin peptide on the increase of kidney weight/body weight in diabetic mice. Considering the dominant contribution of the tubular compartment in organizing renal mass, we speculate that endostatin peptide might have affected the tubular hypertrophy observed in diabetic nephropathy (42). In addition to its potent angiogenic effect, VEGF-A also augments protein synthesis and hypertrophy in renal proximal tubular epithelial cells (43). Therefore, the inhibitory effect of endostatin peptide on the increase of VEGF-A in diabetic mice might have led to the decreased protein synthesis and hypertrophy in tubular epithelial cells, thus resulting in the reduction of renal mass. Serum levels of VEGF were increased in vehicle-treated diabetic animals, which is consistent with a previous report describing increased circulating VEGF levels in type 1 diabetic patients with diabetic nephropathy compared with a normoalbuminuric group (44). Increase in the level of serum VEGF in diabetic animals was suppressed by endostatin peptide in parallel with immunoblot assay findings. Glomerular podocytes overexpress VEGF in patients with early diabetic nephropathy (45). Immunohisto-
chemical analysis revealed a suppressive effect of endostatin peptide on VEGF expression mainly in podocytes in diabetic mice.

In regard to angiopoietins, the expression of Ang-1 in the renal cortex was not altered in diabetic mice as described previously (14), and treatment with endostatin peptide did not affect its level. In contrast, the expression of Ang-2 was markedly increased in diabetic mice similar to our previous results (14). Treatment with endostatin peptide suppressed the increase of Ang-2 in a dose-dependent manner in diabetic mice, close to the level of nondiabetic mice (endostatin peptide at 5 mg/kg). The level of Tie2 was mildly decreased in diabetic mice compared with nondiabetic mice, and endostatin peptide did not alter the level of Tie2. These results suggest the dominant involvement of changes in the level of ligand protein (Ang-1/2) rather than changes in the level of receptor (Tie2) regarding the effect of endostatin peptide in this model. The marked increase of Ang-2 over Ang-1 in diabetic mice suggests the proangiogenic milieu considering simultaneous upregulation of VEGF-A and the “leaky” condition of capillaries in association with an inflammatory response such as monocyte recruitment. Thus, the inhibitory effect of endostatin peptide on the accumulation...
of monocytes/macrophages might be mediated via regulating Ang-2 in addition to its inhibitory effect on VEGF-A/flk-1 signaling.

Nephrin is a functional molecule of the interpodocyte filtration slit diaphragm. Altered expression of nephrin has been reported in the setting of diabetic nephropathy (15,16,46). Reduction in both mRNA and protein expression of nephrin was associated with proteinuria in diabetic spontaneously hypertensive rats (46). In STZ-induced diabetic rats and in nonobese diabetic mice, redistribution of nephrin from a glomerular epithelial cell pattern to a more central glomerular area, increased nephrin mRNA expression, and loss of nephrin protein into urine were observed (15). In human type 1 and type 2 diabetic nephropathy, redistribution and reduced expression of nephrin in glomeruli were demonstrated (16). In the present study, we observed decreased nephrin mRNA expression in diabetic mice, which is consistent with our recent observation (14). Administration of endostatin peptide resulted in the significant recovery of nephrin mRNA in diabetic mice. Results obtained by immunoblot assay further revealed similar effects of endostatin peptide in restoring nephrin protein expression in diabetic mice. Considering the pivotal role of nephrin in the maintenance of the glomerular filtration barrier, the recovery of nephrin by treatment with endostatin peptide might be associated with decreased albuminuria. The indirect influence of glomerular endothelial cells toward podocytes possibly mediated via secreted factors or alteration on a matrix microenvironment might be involved in this effect. A genetic study using mice with podocyte-specific overexpression or deletion of VEGF-A suggested the importance of an appropriate level of VEGF-A in maintaining a proper glomerular filtration barrier (47). The suggested role of Ang-1 in maintaining glomerular endothelium and regulating the action of VEGF-A on glomerular permselectivity (48) further supports the importance of interaction between glomerular endothelial cells and podocytes in maintaining glomerular structures as well as biological functions. Alternatively, the biological function of endostatin on branching morphogenesis of nonendothelial cells such as renal tubular epithelial cells (49) may suggest the direct effect of endostatin peptide on podocytes, which needs further clarification.

The functional receptor for endostatin is considered to be the α5β1-integrin on endothelial cells (23,50) and other cell surface proteins including glypican, flk-1, and E-selectin, an inducible leukocyte adhesion molecule specifically expressed by endothelial cells (24,33,51). Among these cell surface proteins, E-selectin (52) and flk-1 (6) are upregulated on glomerular endothelial cells in diabetic nephropathy, suggesting the involvement of these proteins in mediating the observed biological function of endostatin peptide. Because critical involvement of α5β1-integrin in mediating the antiangiogenic capacity of endostatin has been reported recently (23), we studied the localization of this cell surface receptor in diabetic mice. Although expression of α5β1-integrin was faintly observed in the glomeruli of nondiabetic control mice, increased expression of α5β1-integrin colocalized with CD31 was observed in the glomeruli of diabetic mice as detected by double immunofluorescence. These results suggest the primary action of endostatin peptide on glomerular endothelial cells mediated via α5β1-integrin in diabetic mice. Therefore, we speculate that endostatin peptide indirectly affects podocytes, leading to decreased expression of VEGF in diabetic mice. We previously reported on the therapeutic efficacy of tumstatin peptide using a diabetic nephropathy model (14). The functional receptor for tumstatin in mediating its antiangiogenic effect is considered to be the αvβ3-integrin on endothelial cells (25,28,53–55). Because tumstatin and endostatin are considered to exert an antiangiogenic effect via distinct endothelial integrin receptors (23) and other receptors, coadministration of endostatin peptide and tumstatin peptide may lead to an additive therapeutic effect on diabetic nephropathy.

Endogenous levels of endostatin in the renal cortex were increased in vehicle-treated diabetic mice and suppressed in endostatin peptide–treated diabetic mice detected by immunoblot assay in parallel with VEGF. These findings suggest the possible feedback regulation to obtain a proper balance between proangiogenic and antiangiogenic factors. Although serum levels of endostatin in vehicle buffer–treated diabetic mice were elevated compared with nondiabetic control mice in parallel with renal endostatin levels, serum levels of endostatin seemed to
partly reflect exogenously administered endostatin peptide, considering the elevated serum levels in the high-dose treatment group compared with the low-dose treatment group. In fact, administration of endostatin peptide in nondiabetic mice resulted in higher serum levels of endostatin compared with untreated nondiabetic mice (data not shown).

In conclusion, we demonstrated that the administration of antiangiogenic endostatin peptide effectively ameliorated alterations in early diabetic nephropathy induced by STZ, in addition to its known effects on treating tumor, rheumatoid arthritis, and neovascular retinopathy. Because glomerular hypertrophy in diabetic nephropathy...


37. Berger AC, Feldman AL, Gnant MF, Kruger EA, Sim BK, Hewitt S, Figg WD,