N-Acetyl-Seryl-Aspartyl-Lysyl-Proline Prevents Renal Insufficiency and Mesangial Matrix Expansion in Diabetic *db/db* Mice

Kazuyuki Shibuya,[1] Keizo Kanasaki,[1] Motohide Isono,[1] Haruhisa Sato,[2] Mitsugu Omata,[3] Toshiro Sugimoto,[1] Shin-ichi Araki,[1] Keiji Isshiki,[1] Atsunori Kashiwagi,[1] Masakazu Haneda,[4] and Daisuke Koya[1]

We have previously reported that *N*-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP), which is a tetrapeptide hydrolyzed by ACE, inhibits the transforming growth factor-β (TGF-β)-induced expression of extracellular matrix proteins via inhibition of the Smad signaling in human mesangial cells. To test in vivo the antifibrotic efficacy of Ac-SDKP, we examined whether long-term Ac-SDKP treatment can prevent renal insufficiency and glomerulosclerosis in diabetic *db/db* mice. Diabetic *db/db* mice or nondiabetic *db/m* mice were treated with Ac-SDKP for 8 weeks using osmotic minipumps. The treatment with Ac-SDKP increased plasma Ac-SDKP concentrations by approximately threefold in both groups but did not affect the blood glucose levels. Histologically, the increased glomerular surface area, mesangial matrix expansion, and overproduction of extracellular matrix proteins in *db/db* mice were significantly inhibited by Ac-SDKP. Furthermore, Ac-SDKP treatment normalized the increased plasma creatinine value in *db/db* mice, whereas the albuminuria in Ac-SDKP–treated *db/db* mice was somewhat decreased as compared with nontreated *db/db* mice, although the difference was not statistically significant. In addition, the nuclear translocation of Smad3 was inhibited by Ac-SDKP. These results demonstrate that long-term Ac-SDKP treatment ameliorates renal insufficiency and glomerulosclerosis in *db/db* mice via inhibition of TGF-β/Smad pathway, suggesting that Ac-SDKP could be useful in the treatment of diabetic nephropathy. *Diabetes* 54:838–845, 2005

Diabetic nephropathy is a leading cause of end-stage renal disease, accounting for ~40% of all new patients requiring renal replacement therapy in many countries. Therapeutic strategies for diabetic kidney disease are thus urgently needed worldwide. In the diabetic state, multiple biochemical mechanisms, such as those involving growth factors and cytokines (1), activation of protein kinase C extracellular-regulated protein kinase pathway (2,3), enhanced polyol pathway (4,5), and altered redox state and oxidative stress (6), have been proposed to be involved in the development of diabetic nephropathy.

Transforming growth factor-β (TGF-β) is a key cytokine that regulates development, cell proliferation, and matrix protein synthesis (7,8). During the glomerular scarring process, TGF-β plays a major role in extracellular matrix protein accumulation (9). TGF-β is upregulated in the kidneys of diabetic animal models (10–12) and patients with diabetic nephropathy (13,14). Indeed, the administration of neutralizing anti–TGF-β antibody ameliorated functional and morphological abnormalities in the kidneys of diabetic animals (10,12). Therefore, downregulation of TGF-β signaling provides a useful therapeutic strategy for inhibiting diabetic kidney disease.

Recent large clinical trials (15–17) clearly demonstrated that treatment with ACE inhibitors improves clinical outcome in patients with progressive renal disease, and it is widely appreciated that ACE inhibitors ameliorate glomerular hypertension by reducing the resistance of efferent arterioles. However, the effect of ACE inhibitors might depend not only on the suppression of the renin-angiotensin system but also on other biochemical effects. *N*-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) is a tetrapeptide normally presenting in human plasma and is exclusively hydrolyzed by ACE. The plasma levels of Ac-SDKP were shown to be increased fivefold after ACE inhibitor treatment in patients (18). We recently reported that Ac-SDKP has an antifibrotic property after demonstrating that Ac-SDKP inhibits TGF-β–induced expressions of plasminogen activator inhibitor-1 and α2 (I) collagen in human mesangial cells. We also found that Ac-SDKP inhibits TGF-β signaling pathway through the suppression of Smad2 and Smad3 activations via nuclear export of Smad7 (19),

From the [1]Department of Medicine, Shiga University of Medical Science, Otsu, Shiga, Japan; the [2]Marutamachi Otowa Hospital, Kyoto, Japan; [3]Daiichi Suntory Biomedical Research, Mishima, Osaka, Japan; and the [4]Second Department of Medicine, Asahikawa Medical College, Asahikawa, Hokkaido, Japan.

Address correspondence and reprint requests to Daisuke Koya, MD, Department of Medicine, Shiga University of Medical Science, Otsu, Shiga, 520-2192, Japan. E-mail: koya@belle.shiga-med.ac.jp.

Received for publication 3 August 2004 and accepted in revised form 7 December 2004.

Ac-SDKP, N-acetyl-seryl-aspartyl-lysyl-proline; BSS, balanced salt solution; PAS, periodic acid/Schiff; TGF-β, transforming growth factor-β.

TABLE 1
Characteristics of the experimental groups of mice

	db/m mice		*db/db* mice	
	BSS	Ac-SDKP	BSS	Ac-SDKP
n	13	12	13	13
Body weight (g)	30.6 ± 0.6	30.9 ± 0.4	59.1 ± 1.8*	59.1 ± 0.8*
Blood glucose (mmol/l)	7.8 ± 0.3	7.6 ± 0.2	23.1 ± 1.4*	22.9 ± 1.3*
Left kidney weight (mg)	171.1 ± 6.2	174.2 ± 3.6	208.0 ± 5.7*	208.8 ± 6.2*
Mean blood pressure (mmHg)	84.5 ± 2.2	85.5 ± 2.4	91.8 ± 2.7†	95.9 ± 2.3*
Plasma Ac-SDKP concentration (nmol/l)	0.85 ± 0.04	3.37 ± 1.22†	1.39 ± 0.13	4.79 ± 1.03‡

Data are means ± SE. *$P < 0.01$ vs. *db/m* mice; †$P < 0.05$ vs. *db/m* mice given BSS; ‡$P < 0.05$ vs. *db/db* mice given BSS.

demonstrating that a novel mechanism is involved in the renoprotective effect of ACE inhibitors.

In this study, we therefore investigated the efficacy of the administration of Ac-SDKP in preventing glomerulosclerosis and renal insufficiency in *db/db* mice, a rodent model of type 2 diabetes. An 8-week course of Ac-SDKP treatment prevented renal insufficiency, excess mesangial expansion, and expression of extracellular matrix proteins. In addition, we ascertained that Ac-SDKP inhibits nuclear translocation of Smad3 in diabetic *db/db* mice in vivo. This study provides the first evidence that Ac-SDKP is a natural peptide that inhibits TGF-β signaling in vivo and is useful as a novel therapeutic strategy for diabetic nephropathy.

RESEARCH DESIGN AND METHODS

Male diabetic *db/db* mice and age-matched nondiabetic *db/m* mice were purchased from CLEA Japan (Tokyo, Japan). At 10 weeks of age, osmotic minipumps (Alzet, Cupertino, CA) were surgically implanted for subcutaneous delivery of 1,000 $\mu g \cdot kg^{-1} \cdot day^{-1}$ Ac-SDKP donated by Daiichi Suntory Biomedical Research (Tokyo, Japan) or balanced salt solution (BSS; saline plus 0.01 N acetic acid). We also tested control tetrapeptide (H-Ser-Leu-Leu-Lys-OH) in parallel. The osmotic pumps were replaced when the mice were 14 weeks of age since each pump lasted only 4 weeks. Body weight, blood pressure, blood glucose, erythrocyte count, and hematocrit were measured every 4 weeks. The blood pressure of conscious mice at a steady state was measured by a programmable tail-cuff sphygmomanometer (BP98-A; Softron, Tokyo, Japan). Blood was collected from the caudal vein of mice into heparin-coated microcapillary tubes. Erythrocytes were counted with hemacytometer (Clay Adams, Becton Dickinson, Parsippany, NJ). Hematocrit percentage was assessed using a hematocrit reader (Terumo, Tokyo, Japan). At 18 weeks of age, individual mice were placed in metabolic cages for 24-h urine collection. The urine samples were stored frozen at −80°C. Mice were anesthetized with pentobarbital, and the blood was collected from the cannula into the left ventricle of the heart, put into a heparinized tube containing captopril (final concentration 10 μmol/l), and centrifuged at 2,000*g* for 15 min at 4°C. The plasma was stored at −80°C until the following assays were performed. The kidneys were perfused with ice-cold PBS and 4% neutral buffered paraformaldehyde through the cannula. The Research Center for Animal Life Science of Shiga University of Medical Science approved all experiments.

Measurement of plasma creatinine and Ac-SDKP. Plasma creatinine concentration was quantified with an enzymatic assay kit (Bio Quant, San Diego, CA), and plasma Ac-SDKP concentration was quantified with a competitive enzyme immunoassay kit (SPI-BIO, Massy, France) according to the instructions of the manufacturers.

Urine albumin assays. The urine samples were centrifuged at 2,000*g* for 20 min, and the supernatants were measured with indirect competitive ELISA kits (Exocell, Philadelphia, PA). The urinary albumin excretion was expressed as the total amount excreted in 24 h.

Measurement of renal TGF-β1. The renal cortex was homogenized with ice-cold PBS containing 0.05% Tween 20. The homogenates were centrifuged at 9,000*g* for 15 min at 4°C, and the supernatants were used for measuring renal TGF-β1 by ELISA kits (R&D Systems, Minneapolis, MN).

Protein extraction and Western blot analysis for Smad3. The renal cortex was homogenized and lysed with hypotonic buffer (10 mmol/l HEPES-KCl, pH 7.9, 1 mmol/l EDTA, 15 mmol/l KCl, 2 mmol/l $MgCl_2$, 1 mmol/l dithiothreitol, and protease inhibitor cocktail [Boehringer Mannheim, Lewes, U.K.]) with 0.8% Nonidet P-40, and the lysates were centrifuged at 3,000*g* for 10 min. The supernatants were used as cytosolic extracts, and the pellets were resuspended in high-salt buffer (hypotonic buffer with 420 mmol/l NaCl and 25% glycerol), rotated for 30 min at 4°C, and centrifuged at 17,000*g* for 30 min. The supernatants were used as nuclear extracts. These samples were measured by Western blot analysis, as previously described (19). The electrophoresis was performed on 10% SDS–polyacrylamide gels. Anti-Smad2/3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-nucleoporin p62 antibody (Transduction Laboratories, Lexington, KY) for protein control of nucleus and anti–glyceraldehyde-3-phospate dehydrogenase antibody (Biogenesis, Poole, Dorset, U.K.) for cytosol were used.

Glomerular histology and morphology. Fixed kidneys were embedded in paraffin, sectioned (3 μm), and stained with periodic acid/Schiff (PAS) reagent. Coded sections were read in a double-blind manner by two independent observers. Thirty glomeruli, cut at the vascular pole, were randomly selected from each animal, and the extent of extracellular mesangial matrix was identified by PAS-positive material in the mesangium by using a computer-assisted color image analyzer (LUZEX F; Nikon, Tokyo, Japan) and factored by the glomerular tuft area.

Immunohistochemical analysis. Paraffin-embedded sections were used for immunohistochemical studies of fibronectin, type IV collagen, and Smad3, with anti-mouse fibronectin antibody (A852/R5H; Biogenesis, Poole, England), anti-mouse collagen IV antibody (Becton Dickinson Labware, Bedford, MA), anti-Smad3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and anti–TGF-β type II receptor antibody (Santa Cruz Biotechnology), respectively. Immunostaining was performed by the streptavidin-biotin immunoperoxidase method using a histofine SAB-PO kit (Nichirei, Tokyo, Japan) according to the instructions of the manufacturer. To evaluate the immunostaining of fibronectin and type IV collagen, 20 randomly chosen glomeruli per mouse were coded and graded semiquantitatively in a double-blind manner by two independent observers. The degrees of fibronectin and type IV collagen expression in six mice from each group were graded as follows: 0 = absent staining to 5%, 1 = 5–25%, 2 = 25–50%, 3 = 50–75%, and 4 = >75% (20,21). To evaluate the activation of Smad pathway, >300 cells in each randomly chosen glomerulus of renal outer cortex per mouse were counted, and the number of cells with Smad3 nuclear staining was calculated using a computer-assisted color image analyzer. The Smad3 activation was expressed as the percentage of the distribution of cells with Smad3 nuclear staining.

Statistical analysis. Results are expressed as means ± SE, with *n* denoting the number of animals. ANOVA followed by Scheffe's test was used to determine the significance of difference in multiple comparisons. Correlations between analyses were determined by linear regression analysis. $P < 0.05$ is defined as statistically significant.

RESULTS

Characteristics of experimental mice. The characteristics of the four groups of mice at the end of the experimental period are presented in Table 1. The body weights of the *db/db* mice were significantly heavier than those of the *db/m* mice, and their blood glucose levels were significantly higher. The treatment with Ac-SDKP in both *db/db* and *db/m* mice increased plasma Ac-SDKP levels by approximately threefold compared with BSS-treated mice but did not affect body weights, glucose levels, and mean blood pressures. The kidney weights of *db/db* mice were significantly greater than those of *db/m* mice, and there

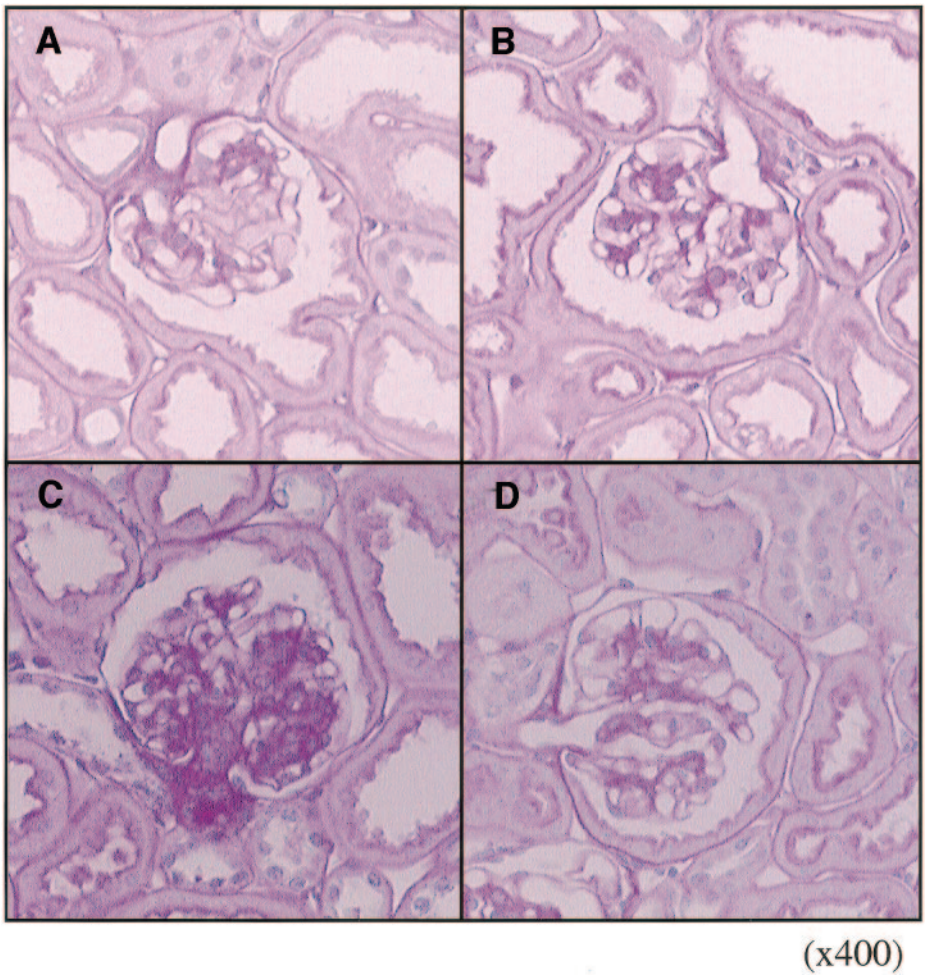


FIG. 1. Ac-SDKP treatment ameliorates mesangial matrix expansion in diabetic *db/db* mice. Representative photomicrographs of PAS-stained kidney sections from *db/m* with BSS (*A*), *db/m* with Ac-SDKP (*B*), *db/db* with BSS (*C*), and *db/db* with Ac-SDKP (*D*).

was no significant difference between BSS- and Ac-SDKP–treated mice.

Since Ac-SDKP was shown to inhibit hematopoiesis (22,23), we evaluated whether the treatment with Ac-SDKP affects peripheral erythrocyte number and the percentage of hematocrit. Erythrocyte counts and hematocrits were not different in *db/m* mice treated with BSS ($12.0 \pm 0.7 \times 10^6$/mm^3, $51.4 \pm 2.3\%$), *db/m* mice with Ac-SDKP ($11.8 \pm 0.9 \times 10^6$/mm^3, $52.7 \pm 1.5\%$), *db/db* mice with BSS ($11.8 \pm 0.9 \times 10^6$/mm^3, $52.0 \pm 0.3\%$), and *db/db* mice with Ac-SDKP ($12.2 \pm 0.3 \times 10^6$/mm^3, $51.6 \pm 0.5\%$).

Glomerular histology. We examined whether treatment with Ac-SDKP attenuated the glomerular hypertrophy and mesangial matrix expansion of diabetic *db/db* mice. The area of PAS-positive matrix in the glomeruli of *db/db* mice was increased compared with that of *db/m* mice. The *db/db* mice treated with Ac-SDKP showed minimal mesangial matrix expansion as compared with the *db/db* mice treated with BSS (Fig. 1). The glomerular surface area was greater in *db/db* than *db/m* mice, and Ac-SDKP treatment significantly attenuated the glomerular hypertrophy in *db/db* mice (Fig. 2*A*). The mesangial matrix expansion was

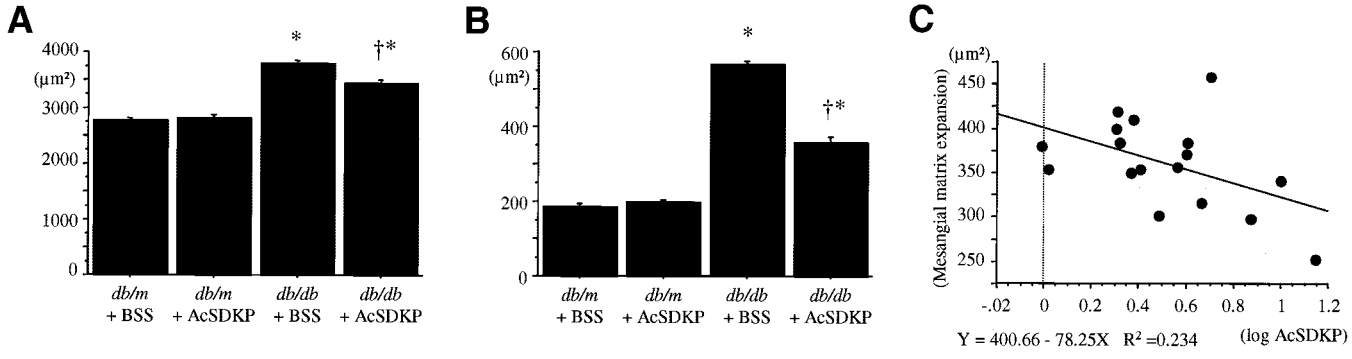


FIG. 2. Ac-SDKP treatment ameliorates glomerular hypertrophy and mesangial matrix expansion in diabetic *db/db* mice. Quantitative measurements of glomerular surface area (*A*) and mesangial matrix area (*B*). *C*: Plasma Ac-SDKP level significantly correlates with mesangial matrix expansion ($r = 0.48$, $P < 0.05$). The graph shows logarithmic expression of Ac-SDKP concentration for the horizontal axis and mesangial matrix expansion for the vertical axis in *db/db* mice treated with Ac-SDKP. Data are means ± SE, $n = 12$ for *db/m* group treated with Ac-SDKP and $n = 13$ for each other group. *$P < 0.01$ vs. *db/m* groups; †$P < 0.01$ vs. *db/db* group treated with BSS.

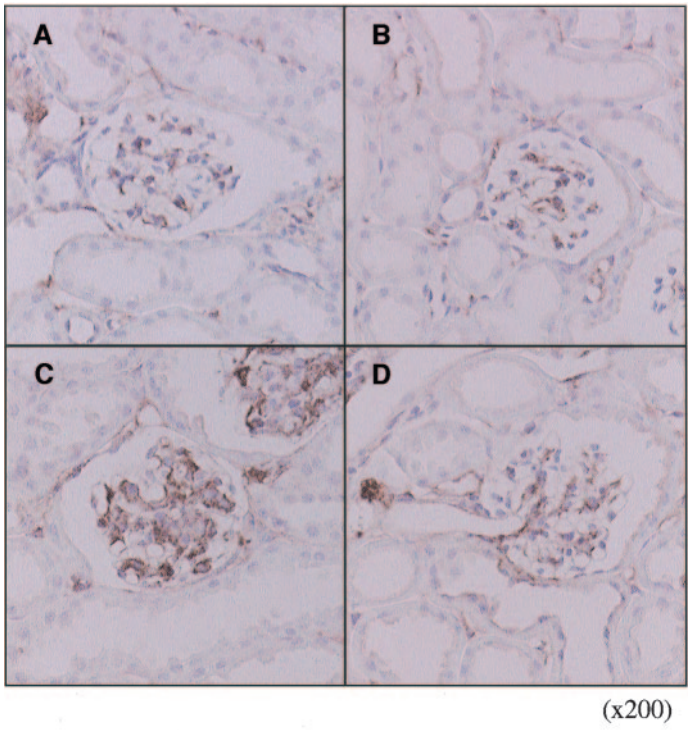


FIG. 3. The treatment with Ac-SDKP prevents the increase in fibronectin expression in *db/db* mice. Representative photomicrographs from *db/m* with BSS (*A*), *db/m* with Ac-SDKP (*B*), *db/db* with BSS (*C*), and *db/db* with Ac-SDKP (*D*). *E*: Semiquanititative scores for fibronectin expression. Data are means ± SE, $n = 6$ for each group. *$P < 0.01$ vs. *db/m* groups; †$P < 0.01$ vs. *db/db* group treated with BSS.

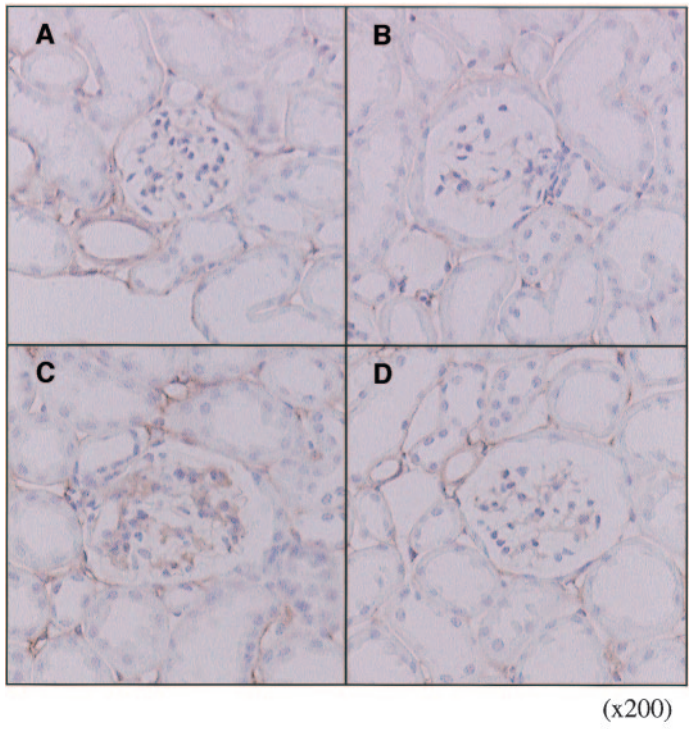


FIG. 4. The treatment with Ac-SDKP prevents the increase in type IV collagen expression in *db/db* mice. Representative photomicrographs from *db/m* with BSS (*A*), *db/m* with Ac-SDKP (*B*), *db/db* with BSS (*C*), and *db/db* with Ac-SDKP (*D*). *E*: Semiquanititative scores for type IV collagen expression. Data are means ± SE, $n = 6$ for each group. *$P < 0.01$ vs. *db/m* groups; †$P < 0.01$ vs. *db/db* group treated with BSS.

increased more than twofold in *db/db* compared with *db/m* mice. Ac-SDKP treatment significantly attenuated the increase in *db/db* mice (Fig. 2*B*). Since the glomerular surface area and mesangial matrix expansion in control tetrapeptide-treated groups were similar to those in BSS-treated groups regardless of their strains (data not shown), we used the BSS treatment as a control in all experiments. Simple regression analysis performed to investigate the effect of Ac-SDKP on the mesangial matrix expansion in *db/db* mice revealed that the treatment with Ac-SDKP decreased the mesangial matrix area in a dose-dependent manner. There was a significant correlation between the mesangial matrix area and the plasma Ac-SDKP level (Fig. 2*C*).

Expression of fibronectin and type IV collagen. The long-term Ac-SDKP treatment successfully prevented the mesangial matrix expansion in *db/db* mice. To evaluate the extracellular matrix components of the mesangial matrix, we performed immunohistochemical studies of fibronectin and type IV collagen. Consistent with the results for mesangial matrix expansion, the diabetic *db/db* mice demonstrated overexpression of fibronectin and type IV collagen as compared with *db/m* mice (Fig. 3 and Fig. 4). Administration of Ac-SDKP also reduced the overexpression of fibronectin and type IV collagen in the glomeruli of *db/db* mice. As shown in Fig. 3*E* and Fig. 4*E*, semiquanititative scores for fibronectin and type IV collagen expression increased from 1.38 ± 0.05 and 1.04 ± 0.06 in *db/m* mice to 3.10 ± 0.04 and 2.90 ± 0.04 in *db/db* mice, respectively. The treatment with Ac-SDKP reduced scores for fibronectin and type IV collagen expression to 2.21 ± 0.08 and 2.16 ± 0.11, respectively.

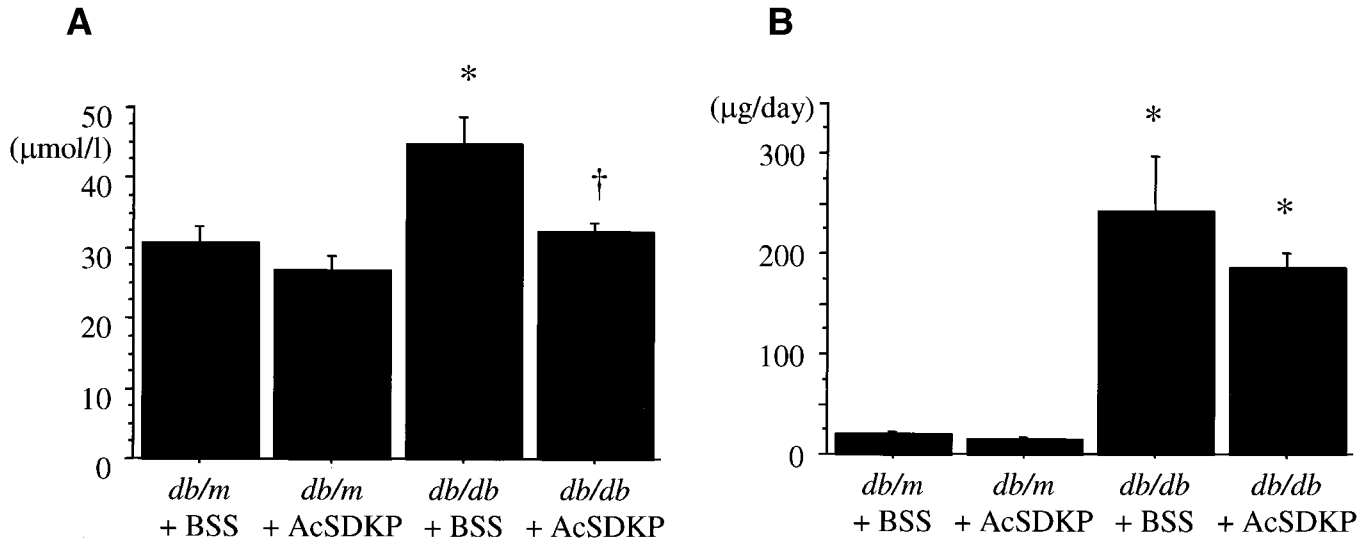


FIG. 5. The treatment with Ac-SDKP prevents the elevation of the plasma creatinine values (*A*) of the *db/db* mice but does not normalize albuminuria (*B*) in *db/db* mice. Data are means ± SE, $n = 12$ for *db/m* group treated with Ac-SDKP and $n = 13$ for each other group. *$P < 0.01$ vs. *db/m* groups; †$P < 0.05$ vs. *db/db* group treated with BSS.

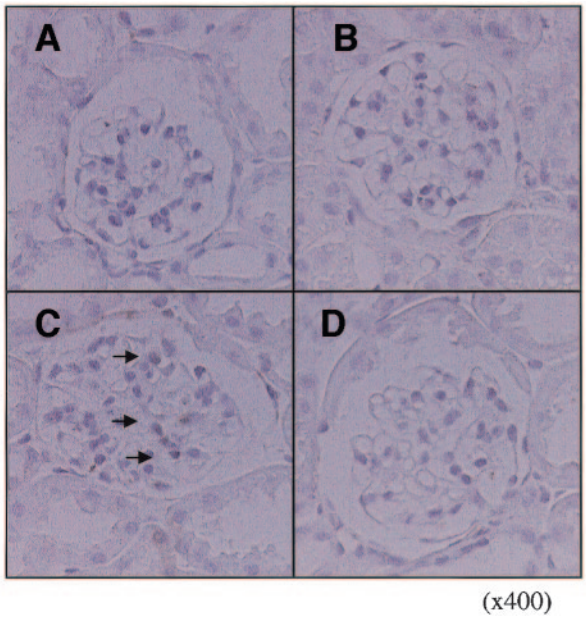
Plasma creatinine and urinary albumin excretion. To evaluate the effect of Ac-SDKP on functional abnormalities in *db/db* mice, the plasma creatinine concentrations and urinary albumin excretions were examined. The plasma creatinine levels were higher in *db/db* than in *db/m* mice, but the treatment with Ac-SDKP normalized the elevation (Fig. 5*A*). The urinary albumin excretion of the BSS-treated *db/db* mice was ~10-fold that of the *db/m* mice. The urinary albumin excretion in the Ac-SDKP–treated *db/db* mice was somewhat decreased compared with that in the BSS-treated *db/db* mice, but the difference was not statistically significant (Fig. 5*B*).

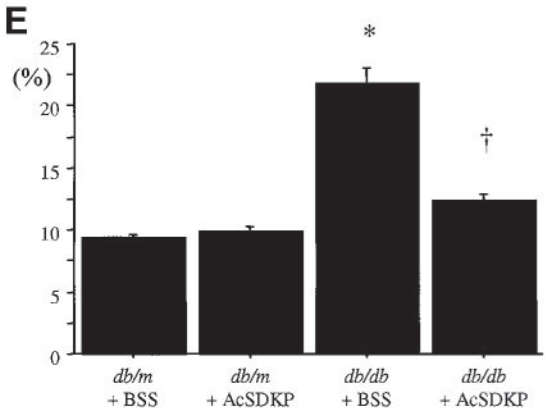
The effect of Ac-SDKP on TGF-β signaling. We previously reported that Ac-SDKP inhibits TGF-β signaling via Smad pathway in mesangial cells (19). To ascertain whether this inhibitory effect is operative in vivo, we performed an immunohistochemical study and Western blot analysis for Smad3 (Fig. 6). Since activated Smad3 was shown to translocate into the nucleus, the activation of Smad3 was evaluated by counting cells with nuclear staining using a computer-assisted color image analyzer. The nuclear translocation of Smad3 in glomeruli of the outer cortex was increased in *db/db* mice (21.7 ± 1.4%) compared with *db/m* mice (9.3 ± 0.4%). The treatment with Ac-SDKP attenuated the increase in the *db/db* mice (12.4 ± 0.5%). Furthermore, the similar result was obtained by Western blot analysis (Fig. 6*F*). These observations demonstrated that Ac-SDKP inhibited TGF-β/Smad signaling in vivo.

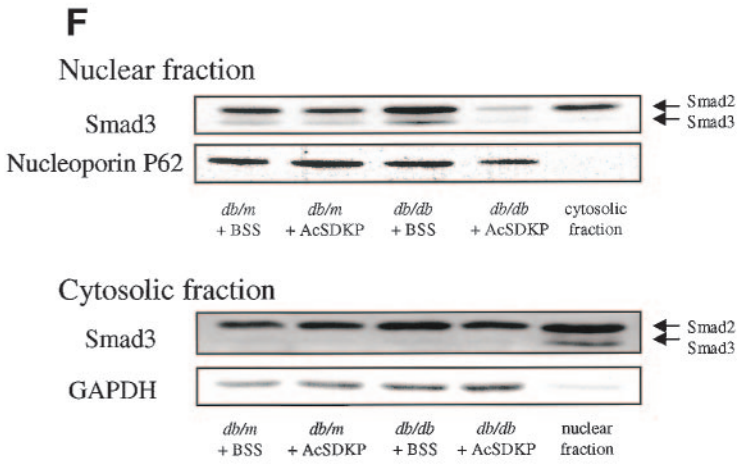
Expression of TGF-β1 and TGF-β type II receptor. It is reported that renal expression of TGF-β1 and TGF-β type II receptor were increased in *db/db* mice (24). To evaluate the effect of Ac-SDKP on renal expression of TGF-β1 and its type II receptor, we performed TGF-β1 enzyme-linked immunosorbent assay and the immunohistochemistry of TGF-β type II receptor. The renal expression of TGF-β1 was increased in *db/db* mice compared with *db/m* mice (95.1 ± 4.3 vs. 79.2 ± 1.7 ng/g protein, $P < 0.05$). The treatment with Ac-SDKP did not change the expression of TGF-β1 in *db/db* mice (91.5 ± 4.5 ng/g protein). Interestingly, the expression of TGF-β type II receptor was upregulated in *db/db* mice, and the treatment with Ac-SDKP inhibited the expression in *db/db* mice (Fig. 7).

DISCUSSION

Growing evidence clearly indicates that TGF-β plays a central role in the pathogenesis of diabetic nephropathy, and the inhibition of TGF-β activity is a powerful strategy in treating diabetic kidney diseases (10,12,25). From this view, we focus on Ac-SDKP, which is hydrolyzed by ACE and shows increased plasma levels in individuals taking ACE inhibitors (18). Our previous study (19) clearly demonstrated that Ac-SDKP can inhibit the TGF-β1–induced plasminogen activator inhibitor-1 and α2 (I) collagen mRNA expression via inhibition of the Smad activation. Therefore, we hypothesized that in vivo Ac-SDKP treatment could have a favorable effect on the progression of diabetic nephropathy. In this study, the 8-week treatment with Ac-SDKP of *db/db* mice prevented extracellular matrix overproduction, mesangial matrix expansion, glomerular hypertrophy, and elevation of plasma creatinine values in parallel with significant increases in plasma Ac-SDKP levels despite persistent hyperglycemia. Although we measured plasma creatinine by using enzymatic assay, we should note that plasma creatinine measured by colorimetric method overestimates true creatinine values in mice (26). To evaluate plasma creatinine more accurately, a high-performance liquid chromatography method would be reliable. Furthermore, plasma Ac-SDKP concentration had a significant correlation with mesangial area (see Fig. 2*C*). This suggests that increasing the concentration of Ac-SDKP can prevent mesangial matrix expansion more completely. On the other hand, albuminuria in *db/db* mice was not significantly affected by the treatment with Ac-SDKP, although it was slightly diminished. In contrast, in a reported study (12) neutralizing anti–TGF-β antibodies failed to diminish albuminuria at all. The discrepancy suggests that Ac-SDKP may possess some unknown function in addition to the inhibition of TGF-β signaling,







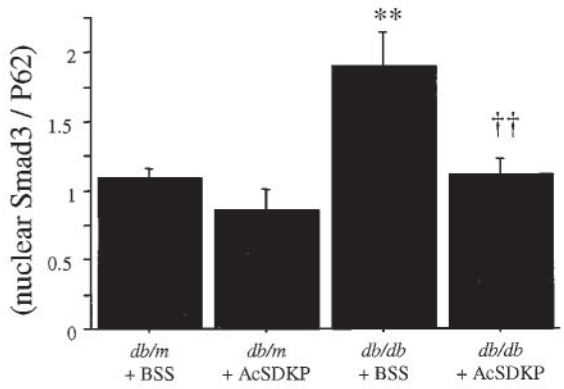


FIG. 6. The treatment with Ac-SDKP inhibits Smad3 nuclear translocation in *db/db* mice. Representative photomicrographs from *db/m* with BSS (*A*), *db/m* with Ac-SDKP (*B*), *db/db* with BSS (*C*), and *db/db* with Ac-SDKP (*D*). *E*: Quantitative results for cells with Smad3 nuclear staining (arrow) in glomeruli. *F*: Nuclear and cytosolic fractions of Smad3 by Western blot analysis and the quantitative results. Data are means ± SE, $n = 5$ for each group. *$P < 0.01$ vs. *db/m* groups; **$P < 0.05$ vs. *db/m groups*; †$P < 0.01$ vs. *db/db* group treated with BSS; ††$P < 0.05$ vs. *db/db* group treated with BSS.

although further studies are needed to elucidate the role of TGF-β in the development of albuminuria.

We next demonstrated that the nuclear translocations of Smad3 were greater in *db/db* than in *db/m* mice by using immunohistochemical studies and Western blot analysis. These results are consistent with a previous report (24) showing increased nuclear accumulation of Smad3 and nuclear binding activity of Smad-binding element in renal glomeruli and tubules of *db/db* mice. The important role of Smad signaling in diabetic kidney disease is evident from the fact that Smad3-null diabetic mice do not exhibit glomerular basement membrane thickening and overexpression of extracellular matrix (27). The treatment with Ac-SDKP significantly attenuated the increase of Smad3 nuclear translocation in *db/db* mice, suggesting that Ac-SDKP inhibited glomerulosclerosis and renal insufficiency through the inhibition of Smad activation in diabetic mice.

We think that Ac-SDKP would be a safe and useful therapeutic agent for several reasons. Firstly, Ac-SDKP is already normally present in the living body, and its level in plasma is increased by ACE inhibitor administration. No harmful effect such as anemia was noted at the dose used in this study. Secondly, ours and other groups (28–30) have reported that several compounds and drugs, such as aminoguanidine, glycosaminoglycan, and thiazolidinediones, prevented diabetic nephropathy via the inhibition of TGF-β expression in diabetic animal models, but Ac-SDKP inhibits TGF-β/Smad signaling specifically. Findings recently reported by Benigni et al. (31) support this notion, since the addition of anti–TGF-β antibody to ACE inhibitors completely arrests proteinuria and renal injury in diabetic rats. Furthermore, in our preliminary study, the combination treatment with Ac-SDKP and captopril increased the plasma Ac-SDKP levels by approximately eightfold and revealed stronger effect on reducing mesangial expansion in db/db mice than Ac-SDKP alone (data not shown).

Diabetic nephropathy is a leading cause of end-stage renal disease, accounting for ~40% of all new admissions for renal replacement therapy in many countries. The morbidity and mortality associated with diabetic nephropathy are extremely high, but the recommended therapies

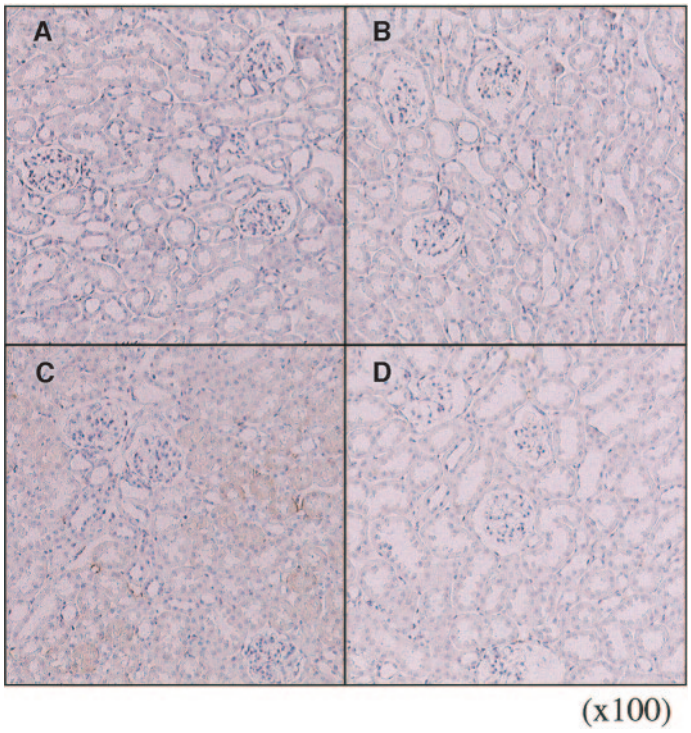


FIG. 7. The treatment with Ac-SDKP prevents the increase in TGF-β type II receptor expression in *db/db* mice. Representative photomicrographs from *db/m* with BSS (*A*), *db/m* with Ac-SDKP (*B*), *db/db* with BSS (*C*), and *db/db* with Ac-SDKP (*D*).

with strict glycemic and blood pressure control decrease them significantly. Since these therapies nevertheless fail to completely prevent the progression of diabetic nephropathy, therapeutic strategies for diabetic kidney disease are urgently needed. Treatment with Ac-SDKP, which demonstrates specific and secure efficacy of inhibition of TGF-β/Smad signaling, may represent a useful therapy for patients with diabetic nephropathy.

ACKNOWLEDGMENTS

This study was supported by a research grant from Takeda Science Foundation, Suzuken Memorial Foundation and the Japanese Ministry of Education, Culture, Sports, Science and Technology (to D.K.).

REFERENCES

1. Sharma K, Ziyadeh FN: Hyperglycemia and diabetic kidney disease: the case for transforming growth factor-β as a key mediator. *Diabetes* 44: 1139–1146, 1995
2. Koya D, King GL: Protein kinase C activation and the development of diabetic complications. *Diabetes* 47:859–866, 1998
3. Haneda M, Araki S, Togawa M, Sugimoto T, Isono M, Kikkawa R: Mitogen-activated protein kinase cascade is activated in glomeruli of diabetic rats and glomerular mesangial cells cultured under high glucose conditions. *Diabetes* 46:847–853, 1997
4. Kikkawa R, Umemura K, Haneda M, Arimura T, Ebata K, Shigeta Y: Evidence for existence of polyol pathway in cultured rat mesangial cells. *Diabetes* 36:240–243, 1987
5. Greene DA, Lattimer SA, Sima AA: Sorbitol, phosphoinositides, and sodium-potassium-ATPase in the pathogenesis of diabetic complications. *N Engl J Med* 316:599–606, 1987
6. Brownlee M: Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813–820, 2001
7. Border WA, Noble NA: Mechanisms of disease: transforming growth factor (beta) in tissue fibrosis. *N Engl J Med* 331:1286–1292, 1994
8. Miyazono K: TGF-beta signaling by Smad proteins. *Cytokine Growth Factor Rev* 11:15–22, 2000
9. Border WA: Transforming growth factor-beta and the pathogenesis of glomerular diseases. *Curr Opin Nephrol Hypertens* 3:54–58, 1994
10. Sharma K, Jin Y, Guo J, Ziyadeh FN: Neutralization of TGF-beta by anti-TGF-beta antibody attenuates kidney hypertrophy and the enhanced extracellular matrix gene expression in STZ-induced diabetic mice. *Diabetes* 45:522–530, 1996
11. Yamamoto T, Nakamura T, Noble NA, Ruoslahti E, Border WA: Expression of transforming growth factor beta is elevated in human and experimental diabetic nephropathy. *Proc Natl Acad Sci U S A* 90:1814–1818, 1993
12. Ziyadeh FN, Hoffman BB, Han DC, Iglesias-De La Cruz MC, Hong SW, Isono M, Chen S, McGowan TA, Sharma K: Long-term prevention of renal insufficiency, excess matrix gene expression, and glomerular mesangial matrix expansion by treatment with monoclonal antitransforming growth factor-beta antibody in db/db diabetic mice. *Proc Natl Acad Sci U S A* 97:8015–8020, 2000
13. Yamamoto T, Noble NA, Cohen AH, Nast CC, Hishida A, Gold LI, Border WA: Expression of transforming growth factor-beta isoforms in human glomerular diseases. *Kidney Int* 49:461–469, 1996
14. Iwano M, Kubo A, Nishino T, Sato H, Nishioka H, Akai Y, Kurioka H, Fujii Y, Kanauchi M, Shiiki H, Dohi K: Quantification of glomerular TGF-beta 1 mRNA in patients with diabetes mellitus. *Kidney Int* 49:1120–1126, 1996
15. The GISEN Group: Randomised placebo-controlled trial of effect of ramipril on decline in glomerular filtration rate and risk of terminal renal failure in proteinuric, non-diabetic nephropathy: the GISEN Group (Gruppo Italiano di Studi Epidemiologici in Nefrologia). *Lancet* 349:1857–1863, 1997
16. Maschio G, Alberti D, Locatelli F, Mann JF, Motolese M, Ponticelli C, Ritz E, Janin G, Zucchelli P: Angiotensin-converting enzyme inhibitors and kidney protection: the AIPRI trial: the ACE Inhibition in Progressive Renal Insufficiency (AIPRI) Study Group. *J Cardiovasc Pharmacol* 33:S16–S20, 1999
17. Ruggenenti P, Perna A, Gherardi G, Gaspari F, Benini R, Remuzzi G: Renal function and requirement for dialysis in chronic nephropathy patients on long-term ramipril: REIN follow-up trial: Gruppo Italiano di Studi Epidemiologici in Nefrologia (GISEN): Ramipril Efficacy in Nephropathy. *Lancet* 352:1252–1256, 1998
18. Azizi M, Rousseau A, Ezan E, Guyene TT, Michelet S, Grognet JM, Lenfant M, Corvol P, Menard J: Acute angiotensin-converting enzyme inhibition increases the plasma level of the natural stem cell regulator N-acetyl-seryl-aspartyl-lysyl-proline. *J Clin Invest* 97:839–844, 1996
19. Kanasaki K, Koya D, Sugimoto T, Isono M, Kashiwagi A, Haneda M: N-Acetyl-seryl-aspartyl-lysyl-proline inhibits TGF-beta-mediated plasminogen activator inhibitor-1 expression via inhibition of Smad pathway in human mesangial cells. *J Am Soc Nephrol* 14:863–872, 2003
20. Hugo C, Hugo C, Pichler R, Gordon K, Schmidt R, Amieva M, Couser WG, Furthmayr H, Johnson RJ: The cytoskeletal linking proteins, moesin and radixin, are upregulated by platelet-derived growth factor, but not basic fibroblast growth factor in experimental mesangial proliferative glomerulonephritis. *J Clin Invest* 97:2499–2508, 1996
21. Koya D, Haneda M, Nakagawa H, Isshiki K, Sato H, Maeda S, Sugimoto T, Yasuda H, Kashiwagi A, Ways D, King G, Kikkawa R: Amelioration of accelerated diabetic mesangial expansion by treatment with a PKC beta inhibitor in diabetic db/db mice, a rodent model for type 2 diabetes. *FASEB J* 14:439–447, 2000
22. Le Meur Y, Lorgeot V, Comte L, Szelag JC, Aldigier JC, Leroux-Robert C, Praloran V: Plasma levels and metabolism of AcSDKP in patients with chronic renal failure: relationship with erythropoietin requirements. *Am J Kidney Dis* 38:510–517, 2001
23. Grillon C, Rieger K, Bakala J, Schott D, Morgat JL, Hannappel E, Voelter W, Lenfant M: Involvement of thymosin beta 4 and endoproteinase Asp-N in the biosynthesis of the tetrapeptide AcSerAspLysPro a regulator of the hematopoietic system. *FEBS Lett* 274:30–34, 1990
24. Hong SW, Isono M, Chen S, Iglesias-De La Cruz MC, Han DC, Ziyadeh FN: Increased glomerular and tubular expression of transforming growth factor-beta1, its type II receptor, and activation of the Smad signaling pathway in the db/db mouse. *Am J Pathol* 158:1653–1663, 2001
25. Wolf G, Ziyadeh FN: Molecular mechanisms of diabetic renal hypertrophy. *Kidney Int* 56:393–405, 1999
26. Dunn SR, Qi Z, Bottinger EP, Breyer MD, Sharma K: Utility of endogenous creatinine clearance as a measure of renal function in mice. *Kidney Int* 65:1959–1967, 2004
27. Fujimoto M, Maezawa Y, Yokote K, Joh K, Kobayashi K, Kawamura H, Nishimura M, Roberts AB, Saito Y, Mori S: Mice lacking Smad3 are protected against streptozotocin-induced diabetic glomerulopathy. *Biochem Biophys Res Commun* 305:1002–1007, 2003
28. Kelly DJ, Gilbert RE, Cox AJ, Soulis T, Jerums G, Cooper ME: Aminogua-

nidine ameliorates overexpression of prosclerotic growth factors and collagen deposition in experimental diabetic nephropathy. *J Am Soc Nephrol* 12:2098–2107, 2001
29. Ceol M, Gambaro G, Sauer U, Baggio B, Anglani F, Forino M, Facchin S, Bordin L, Weigert C, Nerlich A, Schleicher ED: Glycosaminoglycan therapy prevents TGF-beta1 overexpression and pathologic changes in renal tissue of long-term diabetic rats. *J Am Soc Nephrol* 11:2324–2336, 2000
30. Isshiki K, Haneda M, Koya D, Maeda S, Sugimoto T, Kikkawa R: Thiazolidinedione compounds ameliorate glomerular dysfunction independent of their insulin-sensitizing action in diabetic rats. *Diabetes* 49:1022–1032, 2000
31. Benigni A, Zoja C, Corna D, Zatelli C, Conti S, Campana M, Gagliardini E, Rottoli D, Zanchi C, Abbate M, Ledbetter S, Remuzzi G: Add-on anti-TGF-beta antibody to ACE inhibitor arrests progressive diabetic nephropathy in the rat. *J Am Soc Nephrol* 14:1816–1824, 2003