Urinary Smad1 Is a Novel Marker to Predict Later Onset of Mesangial Matrix Expansion in Diabetic Nephropathy

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OBJECTIVE—We reported that Smad1 is a key transcriptional factor for mesangial matrix expansion in diabetic nephropathy. In this study, we examined whether urinary Smad1 in an early phase of diabetes can predict later development of glomerulosclerosis in diabetic nephropathy and how an angiotensin II type 1 receptor blocker (ARB) can modulate structural changes and urinary markers.

RESEARCH DESIGN AND METHODS—Smad1 and albumin in the urine were examined 4 weeks after injection of streptozotocin in 48 rats or 6 weeks of diabetes in *db/db* mice. Their renal pathology was analyzed after 20 weeks in rats or 12 weeks in mice. Among 48 diabetic rats 7 rats were treated with olmesartan for 20 weeks.

RESULTS—Urinary Smad1 of diabetic rats at 4 weeks was nicely correlated with mesangial matrix expansion at 24 weeks ($r = 0.70$, $P < 0.001$), while albuminuria showed a weaker association ($r = 0.31$, $P = 0.043$). Olmesartan treatment significantly ameliorated glomerulosclerosis and dramatically decreased urinary Smad1 (from 3.9 ± 2.9 to 0.3 ± 0.3 ng/mg creatinine, $P < 0.05$). In *db/db* mice, urinary Smad1 at 6 weeks was also significantly correlated with mesangial expansion at 18 weeks. In contrast, there was no change in urinary Smad1 in control diabetic rats or mice.

CONCLUSIONS—The increase of urinary Smad1 in the early stages of diabetes is correlated with later development of glomerulosclerosis in two rodent models. These data indicate that urinary Smad1 could be a novel predictor for later onset of morphological changes and can be used to monitor the effect of ARBs in diabetic nephropathy. *Diabetes* 57:1712–1722, 2008

Diabetic nephropathy is a morbid complication associated with diabetes and the most common cause of end-stage renal disease. Diabetic nephropathy is characterized by mesangial matrix expansion followed by glomerulosclerosis, leading to accumulation of extracellular matrix (ECM) components secreted mainly from mesangial cells (1). Accumulating evidence indicates that the administration of angiotensin II type 1 receptor blockers (ARBs) slows the progression of renal disease and delays the need for dialysis or transplantation in diabetic patients (2,3). We have recently reported that Smad1 is a key molecule for direct transcriptional regulation of type IV collagen (Col4) in vitro, which is one of the major components of increased ECM in diabetic nephropathy (4), and that both glomerular expression of Smad1 and urinary excretion of Smad1 are increased significantly in diabetic rats along with mesangial expansion. Moreover, we have demonstrated that ARBs ameliorate the development of diabetic nephropathy through the inhibition of the Src/Smad1 pathway (5).

The term albuminuria first appeared in the literature 40 years ago (6). Thereafter, albuminuria has been used as a predictor of future overt diabetic nephropathy, which is recognized as a progressive stage. Recent evidence (7,8) shows that modulating therapies, including strict blood glucose control, blood pressure control with ARBs or ACE inhibitors, and diet therapy, at the stage of albuminuria can prevent the development of overt nephropathy. Therefore, albuminuria is a clinically useful diagnostic marker for predicting future overt nephropathy, but both sensitivity and specificity of alubuminuria are not high enough for detecting the initial stage of nephropathy.

Although the measurement of albuminuria has been used for estimating renal function in the early phase of diabetic nephropathy, we and others showed that albuminuria, per se, is not always correlated with mesangial matrix expansion during that phase (9,10), which is a key pathological finding for detecting decreased renal function in insulin-dependent diabetes. Other molecules, such as urinary excretion of Col4, transforming growth factor-β, and connective tissue growth factor, are still not satisfactory to detect diabetic glomerulosclerosis (11,12). So far, renal biopsy is the only way to detect the therapeutical effects of diabetic nephropathy, but it is not practical to do a renal biopsy in all diabetic patients. Thus, it is necessary to establish a noninvasive marker reflecting both predictable and therapeutical effects.

Therefore, we examined whether increased urinary Smad1 in the early phase of diabetes is associated with the later onset of mesangial matrix expansion followed by glomerulosclerosis in two rodent diabetic nephropathy
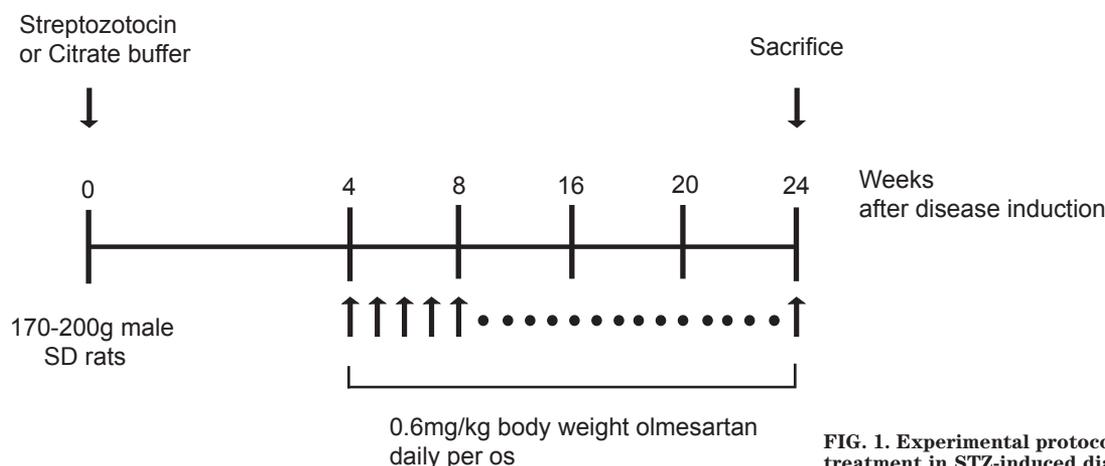
FIG. 1. Experimental protocol of olmesartan treatment in STZ-induced diabetic rats.

models and whether ARBs can ameliorate diabetic structural changes along with urinary Smad1. This study should provide the first evidence that the increase in urinary Smad1 in the early stage of diabetes is correlated with the later development of glomerulosclerosis and that ARB treatment significantly ameliorates glomerulosclerosis, perfectly consistent with a decrease of urinary Smad1. These data indicate that urinary Smad1 could be a novel predictor for future morphological changes and can be used to monitor the effect of ARBs in diabetic nephropathy.

RESEARCH DESIGN AND METHODS

We used age-matched male Sprague-Dawley rats and C57BLKS/J[Lepr] background *db/db*, db/m mice bred at the Shimizu Laboratory Animal center (Hamamatsu, Japan). The animals were housed under specific pathogen-free conditions at the animal facility of Kyoto University, Faculty of Medicine. All of animal experiments were performed in accordance with institutional guidelines, and the review board of Kyoto University granted ethical permission to this study. Diabetes was induced in male rats weighing 170–200 g by a single intravenous injection of streptozotocin (STZ) (Wako, Osaka, Japan) (55 mg/kg body wt) in a 0.05 mol/l citrate buffer (pH 4.5). Rats receiving an injection of citrate buffer were used as controls (5). The levels of blood glucose were determined 2 days after injection of STZ or citrate buffer, and rats with blood glucose levels >16.7 mmol/l were used as the diabetic group. Four weeks after injection of STZ, some rats were killed after examining no morphological changes at this time. The other rats were fed and killed at 24 weeks ($n = 48$). Seven of 48 rats were treated with the ARB, olmesartan (Sankyo, Tokyo, Japan), using sonde (diabetic rats treated with ARB, $n = 7$) for the following 20 weeks (Fig. 1). db/m (control mice, $n = 6$) and *db/db* ($n = 10$) mice were fed and killed at 18 weeks.

Serum creatinine and blood urea nitrogen were measured with a Hitachi Mode 736 autoanalyzer (Hitachi, Tokyo, Japan) (13). The levels of blood glucose and A1C were measured using a One Touch Ultra (Johnson &

Johnson, Tokyo, Japan) and DCA2000 analyzer (Bayer Medical, Tokyo, Japan). In rats, 4 and 24 weeks after injection of STZ, creatinine and albumin were measured from a 24-h urine collection, while blood glucose and A1C were measured in mice aged 6 and 18 weeks.

Renal histology and morphometric analysis
Light microscopy. Methyl Carnoy's solution-fixed, paraffin-embedded kidney sections (2 μm) were stained with periodic acid silver methenamine (PASM) and periodic acid Schiff (PAS). Tissue blocks for light microscopy examination were fixed in methyl Carnoy's solution and embedded in paraffin. Sections (2 μm) were stained with PASM and PAS. Glomerular morphometry was evaluated in PASM-stained tissues. The glomerular surface area and the PASM-positive area/glomerular area (%) were measured using an image analyzer with microscopy (Image Processor for Analytical Pathology; Sumitomo Chemical, Osaka, Japan) as described (5,14–17). For each animal, 50 glomeruli were analyzed.
Immunohistochemistry. Kidney sections were processed for immunohistochemistry following standard procedures. To study Col4, ethyl Carnoy's solution-fixed and paraffin-embedded tissue blocks were used. Kidney sections were rehydrated and treated with 0.3% hydrogen peroxide in methanol for 30 min. To eliminate nonspecific staining, sections were incubated with the appropriate preimmune serum for 60 min at room temperature and then incubated with avidin D and biotin blocking solutions (Vector, Burlingham, CA) for 15 min each. Sections were incubated with anti-Col4 antibody (1:200 dilution) (Progen Biotechnik, Heidelberg, Germany) overnight at 4°C and then incubated with the appropriate biotinylated secondary antibodies followed by incubation with the avidin-biotin peroxidase complex (Vectastain Elite ABC kit; Vector). Peroxidase conjugates were subsequently localized using diaminobenzidine tetrahydrochloride. To quantificate the expression of Col4, frozen kidney sections were used. Sections (4 μm) were fixed in acetone, blocked with 10% donkey serum, and incubated overnight with anti-Col4 antibody (1:200 dilution) (Progen Biotechnik), and the Col4-positive area in glomeruli was measured using Image-Pro PLUS (Media Cybernetics, Silver Spring, MD) as described (5,18). For each animal, 50 glomeruli were evaluated.
Measurement of urinary variables. Urinary albumin and creatinine were measured at 24 weeks from 24-h urine collection samples from rats housed in

TABLE 1
Biochemical parameters in each experimental group

	Control	Diabetes	Diabetes plus olmesartan
n	6	41	7
Body weight (g)	642 ± 31	447 ± 87*	354 ± 49*
Systolic blood pressure (mmHg)	117 ± 3.1	112 ± 5.9	108 ± 6.1
Urine volume (ml/day)	8.5 ± 2.0	150 ± 65*	74 ± 70*
Blood glucose (mg/dl)	113 ± 22	375 ± 103*	394 ± 152*
A1C (%)	2.6 ± 0.1	7.0 ± 1.9*	6.4 ± 0.8*
Albuminuria (mg/day)	1.0 ± 0.5	12 ± 1.3*	4.8 ± 3.0†
Ccr (ml · day^{-1} · 100 g body wt^{-1})	8.3 ± 0.2	26 ± 9.0*	19 ± 3.5†

Data are means ± SD. Control = control rat, Diabetes = STZ-induced diabetic rat with vehicle, Diabetes plus olmesartan = STZ-induced diabetic rats treated with olmesartan. Rats with olmesartan treatment were administered 0.6 mg/kg olmesartan. After 24 weeks of STZ injection, systolic blood pressure was measured by the cuff-tailed method, and the rats were weighed and killed. Blood was taken to evaluate blood glucose and A1C. Ccr was calculated and normalized as described in research design and methods. Albuminuria was measured by Nephrat. *$P < 0.05$ vs. control; †$P < 0.05$ vs. diabetes.

TABLE 2
Biochemical data in each group

	Control	Diabetes
n	6	10
Body weight (g)	32 ± 1.1	54 ± 3.6*
Blood glucose (mg/dl)	75 ± 10	540 ± 103*
A1C (%)	2.6 ± 0.1	9.2 ± 0.8*

Data are means ± SD. Control = db/m control mouse, Diabetes = db/db diabetic mouse. At 18 weeks of age, mice were weighed and killed. Blood was taken to evaluate blood glucose and A1C. *$P < 0.05$ vs. control.

individual metabolic cages. During the urine collection, the rats were allowed free access of to food and water. Albumin concentration in the urine was measured by Nephrat and Albuwell (Exocell, Philadelphia, PA), and urinary excretion of Col4 was measured by Collagen IV M (Exocell), according to the manufacture's protocols. Body weight–adjusted creatinine clearance (Ccr) was calculated by the following equation: Ccr = urine creatinine (mg/dl) × urine volume (μL/min)/serum creatinine (mg/dl)/body wt (g) (5,19).

Recombinant Smad1 protein. cDNA encoding human Smad1 was kindly provided by Dr. K. Miyazono (Tokyo University, Tokyo, Japan). cDNA was subcloned into the prokaryotic expression vector pGEX-4T (Amersham Biosciences, Piscataway, NJ) in frame at the *Bam*HI and *Xho*I site for glutathione *S*-transferase fusion protein. This glutathione *S*-transferase fusion protein was

produced in *E. coli* strain DH5α and purified according to the manufacturers' instructions. The purified recombinant protein was extensively dialyzed against 0.1 mol/l Phosphate buffer and stored at −80°C until use. Protein concentrations were determined by the Bradford method (Bio-Rad) or from the intensities of the bands in Coomassie blue–stained SDS-PAGE gels using BSA as a standard.

Urine preparation. Urine samples were centrifuged at room temperature for 15 min. The supernatants were stored at −80°C and rapidly thawed and centrifuged to remove any urates or phosphates before use in assays. Because Smad1 concentrations in urine are below the detection limits of our assays, the following methods for concentration were developed and tested.

A 2.0-ml urine sample was placed in a Centricon-10 filter (Amicon) pretreated with 0.1% Tween-20 used to limit adsorption to its polypropylene components. The extent of concentration achieved was calculated from the exact retenate volume measured with a Hamilton pipet. This ranged from 25- to 60-fold, with a median of 55-fold concentration. This prepared urine concentrate was diluted to total volume of 110 ml with the dilution buffer as described below. Subsequently, 100 μl of the 110-μl total volumes was utilized in the following assays. The final urine concentration was 18-fold higher than the original sample. This was taken into consideration in the final calculation of the concentration of Smad1 in each urine sample.

Enzyme-linked immunosorbent assay procedure. The following buffers were prepared with deionized water and used in the enzyme-linked immunosorbent assay (ELISA). The coating buffer is 0.1 mol/l phosphate buffer, pH 7.4, containing 3% BSA (Sigma). The dilution buffer is phosphate buffer containing 3% BSA and 0.1% Tween 20. The washing buffer is phosphate buffer containing 0.1% Tween 20.
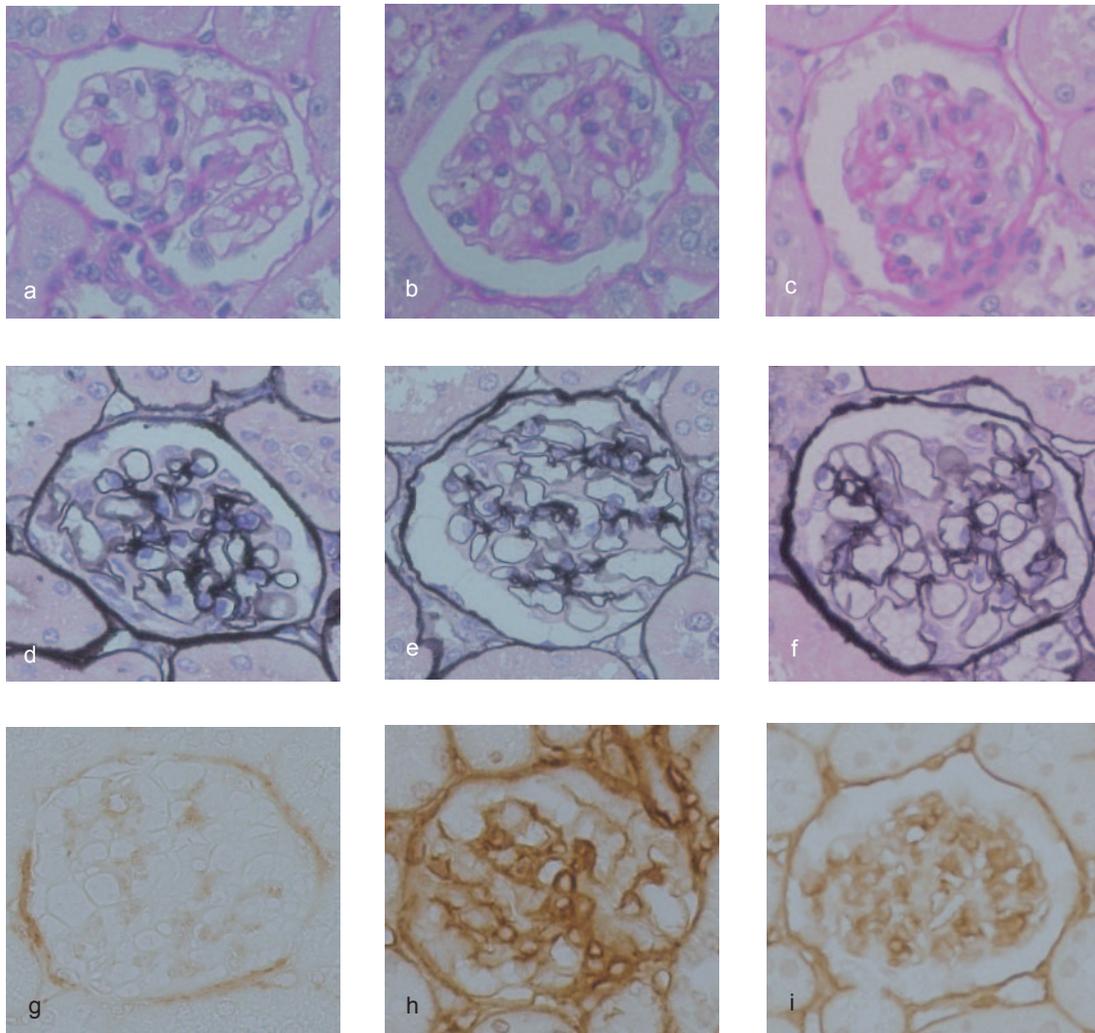

FIG. 2. Glomerular expression of Col4 at 4 weeks after STZ injection. Representative light microscopic appearance of glomeruli (PAS and PASM staining) and immunohistochemistry of Col4 for control rats (*a*, *d*, and *g*), diabetic rats with high urinary Smad1 (*b*, *e*, and *h*), and diabetic rats with low urinary Smad1 (*c*, *f*, and *i*). High urinary Smad1: data from the rats in which urinary Smad1 at 4 weeks was equal or higher than the median. Low urinary Smad1: data from the rats in which urinary Smad1 at 4 weeks was lower than the median. *a–c*: PAS staining. *d–f*: PASM staining. *g–i*: Col4. The original magnification was ×400.
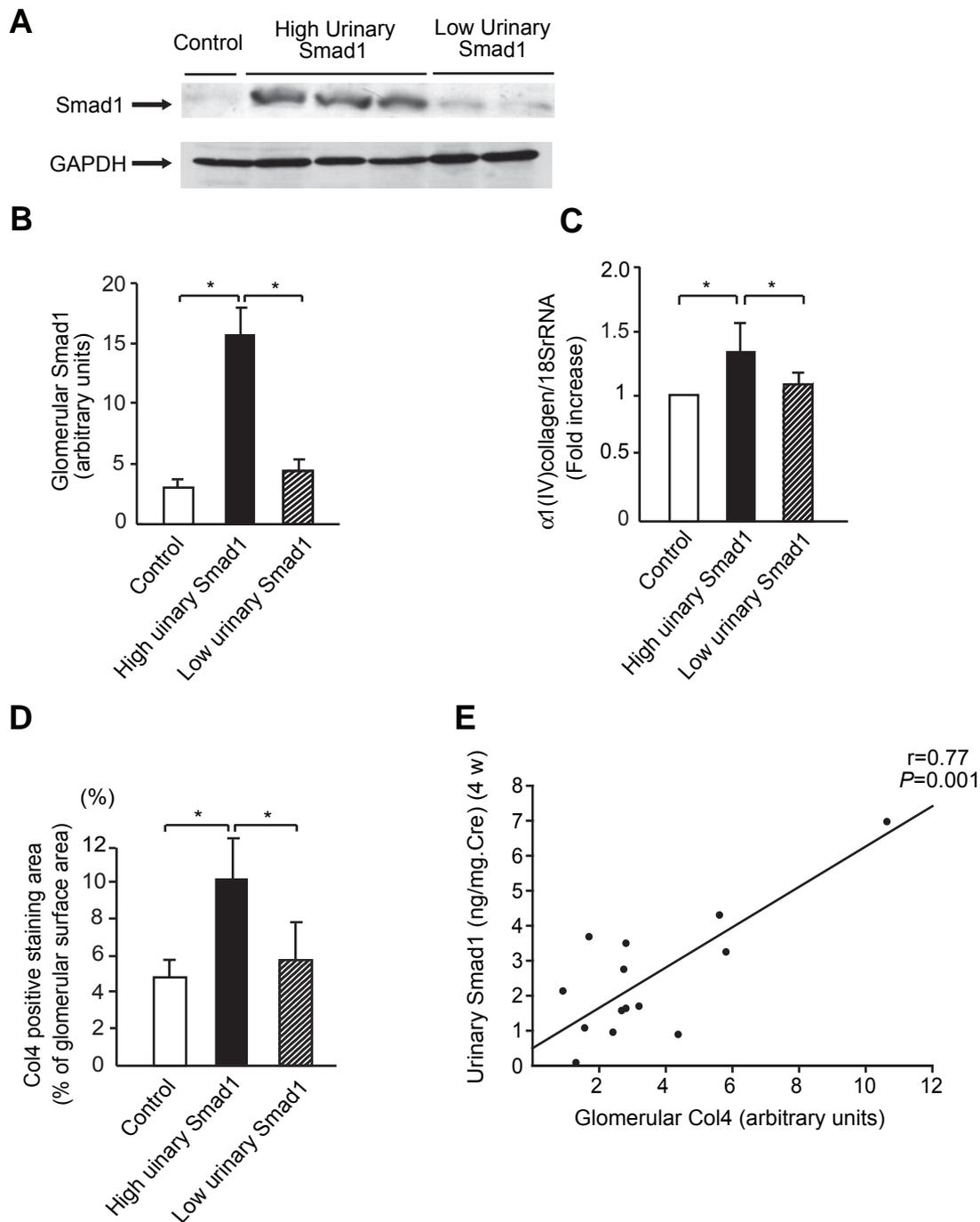
FIG. 3. Glomerular expression of Col4 at 4 weeks after STZ injection. *A*: Immunoblots for Smad1 in control and diabetic rats at 4 weeks are shown. Proteins (20 µg) obtained from each glomerular lysates were loaded. Each lane represents a representative Western blot for the glomerular lysates from each rat. *B*: Quantification of glomerular Smad1 by optical densitometry. The data are expressed as means ± SD. *C*: Glomerular mRNA gene expression for Col4a1 was measured by real-time RT-PCR. The data are expressed as means ± SD. *D*: Morphometric analysis of the glomerular expression of Col4. The glomerular staining area of Col4 was measured as described in RESEARCH DESIGN AND METHODS. The data are expressed as means ± SD. High urinary Smad1: data from the rats in which urinary Smad1 at 4 weeks was equal or higher than the median. Low urinary Smad1: data from the rats in which urinary Smad1 at 4 weeks was lower than the median. *$P < 0.05$. *E*: The correlation between glomerular expression of Col4 (arbitrary units obtained from band intensity of immunoblots) and urinary Smad1.

All the ELISAs were performed in 96-well microtiter plates (Nunc). Anti-Smad1 monoclonal antibody (Santa Cruz) was diluted with the coating buffer to obtain a final concentration of 2.5 mg/ml. Aliquots (250 ng/100 ml) were added to each well, and the plate was incubated overnight at 4°C. The wells were washed three times. Fifty microliters of the prepared urine samples and standard solutions of recombinant Smad1 (prepared at concentrations ranging from 0.125 to 512 ng/ml with the dilution buffer) were added to the plate and incubated overnight at 4°C. The plates were then washed three times. One hundred microliters of rabbit anti-Smad1 polyclonal antibody (Upstate) was diluted with the diluting buffer to obtain a final concentration

of 2.5 mg/ml then added to each well and incubated overnight at 4°C. Horseradish peroxidase–conjugated anti-rabbit IgG polyclonal antibody (Amersham Biosciences) was diluted 500-fold with the dilution buffer, and 50 µl were then dispensed into each well and the plate was further incubated at room temperature for 120 min. The plates were washed three times.

Fifty microliters of freshly prepared peroxidase substrate solution (Dako) were added to each well. The plates were kept at room temperature in the dark for 30 min, and the enzyme reaction was then stopped by adding 50 µl of 2 N H_2SO_4 to each well. The optical density was measured at 492 nm using a microplate reader (Spectra; Tecan, Grödig, Austria). Each Smad1 determina-
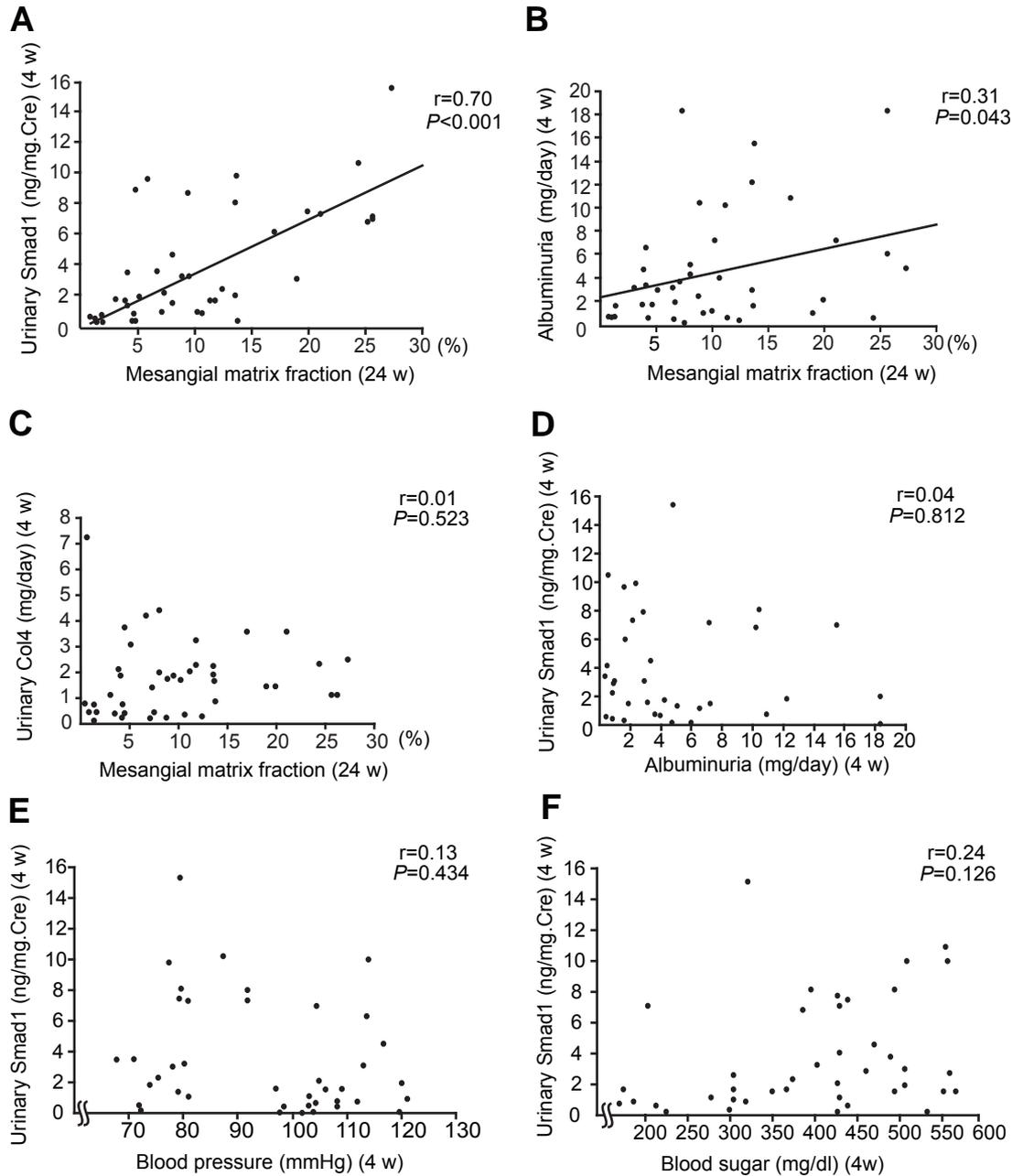
FIG. 4. Association of urinary Smad1 with glomerular expansion in STZ rats. *A*: The correlation between urinary Smad1 at 4 weeks and mesangial matrix fraction at 24 weeks is shown ($r = 0.70, P < 0.001$). Smad1 in two consecutive 24-h urine samples in untreated diabetic rats was measured by ELISA and normalized by the amount of creatinine in the same sample. Correlation between albuminuria at 4 weeks and mesangial matrix fraction at 24 weeks ($r = 0.31, P = 0.043$) (b) or urinary Col4 at 4 weeks ($r = 0.01, P = 0.523$) (*C*). *D*: Correlation between urinary Smad1 and albuminuria at 4 weeks ($r = 0.04, P = 0.812$). *E*: Correlation between urinary Smad1 and blood pressure at 4 weeks ($r = 0.13, P = 0.434$). *F*: Correlation between urinary Smad1 and blood glucose at 4 weeks ($r = 0.24, P = 0.126$).

tion was carried out in duplicate. Delta SOFT3TM (Biometallics) was used to obtain the calibration curve and the polynomial equation. The optical densities of all urine samples were then inserted in the equation in order to calculate the corresponding Smad1 concentration.

Isolation of glomeruli. Rat glomeruli were isolated from the renal cortex of rats using the differential sieving method. The purity of the glomeruli was >90%.

Western blotting. Isolated glomerular mesangial cells were suspended in radioimmunoprecipitation assay buffer (50 mmol/l Tris, pH 7.5; 150 mmol/l NaCl; 1% Nonindet P-40; 0.25% SDS; 1 mmol/l Na_3VO_4; 2 mmol/l EDTA; 1 mmol/l phenylmethylsulfonyl fluoride; and 10 mg/ml of aprotinin) and incubated for 1 h at 4°C. After centrifugation, the supernatants were used as total cell lysates. Twenty micrograms of each sample were applied to SDS-PAGE. After electrophoresis, the proteins were transferred to nitrocellulose filters (Schleicher & Schuell, Keene, NH). The blots were subsequently incubated with anti-Smad1 (Upstate) or anti-Col4 antibody (Progen Biotechnik), fol-

lowed by incubation with horseradish peroxidase–conjugated goat anti-rabbit IgG and sheep anti-mouse IgG (Amersham Biosciences). β-Actin (Cell Signaling Technology, Beverly, MA) or glyceraldehyde 3-phosphate dehydrogenase (Clontech, Mountain View, CA) was used as a loading control. The immunoreactive bands were visualized using horseradish peroxidase–conjugated secondary antibody and the enhanced chemiluminescent system (Amersham Biosciences). These bands were quantificated by imaging densitometer (Science Lab 99 Image Gauge; Fujifilm, Tokyo, Japan).

RNA isolation and quantitative real-time RT-PCR of collagen α1(IV) mRNA. One microgram of total RNA was used to prepare complementary DNA with Superscript III reverse transcriptase (Invitrogen), according to the manufacture's protocol. Real-time PCR was performed to measure the mRNA levels of collagen α1(IV) and 18S rRNA, an internal control, for each sample in separate wells in duplicate on an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA) using the SYBR Green PCR Master Mix (Applied Biosystems). The parameters included a single cycle of
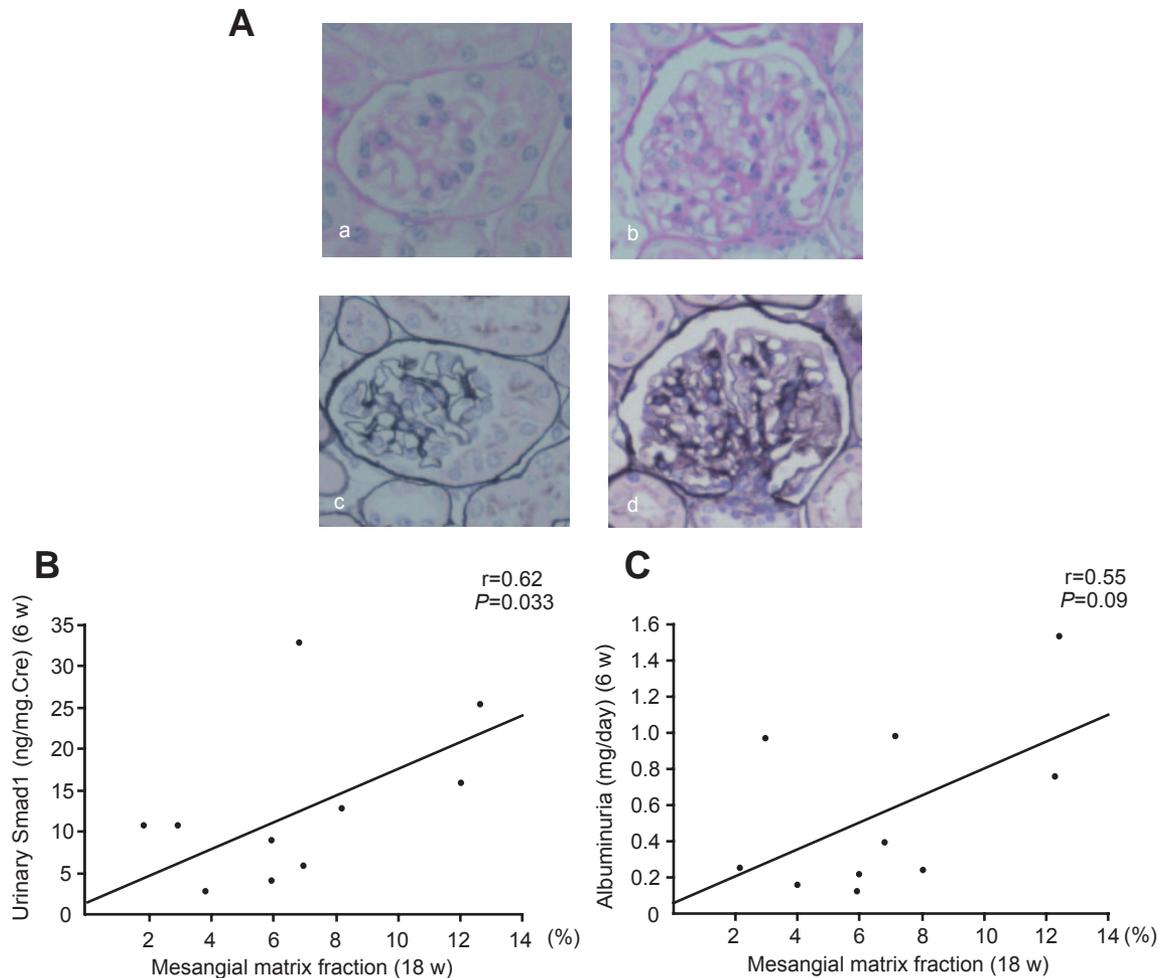
FIG. 5. Association of urinary Smad1 with glomerular expansion in *db/db* mice. A: Representative light microscopic appearance of glomeruli (PAS and PASM staining) for db/m mice (*a* and *c*) and *db/db* mice (*b* and *d*). *a* and *b*: PAS staining. *c* and *d*: PASM staining. The original magnification was ×400. B: The correlation between urinary Smad1 at 6 weeks and mesangial matrix fraction at 18 weeks is shown ($r = 0.62$, $P = 0.033$). C: Correlation between albuminuria and mesangial matrix fraction at 18 weeks ($r = 0.55$, $P = 0.09$).

95°C for 10 min, followed by 40 cycles of 95°C for 15 s, annealing, and 60°C for 1 min. The primers were collagen α1(IV) forward, 5′-ATTCCTTTGTGATGCA CACCAG-3′; collagen α1(IV) reverse, 5′-AAGCTGTAAGCATTCGCGTAGTA-3′; 18S rRNA forward, 5′-CGTTCTTAGTTGGTGGAGCGA-3′; and 18S rRNA reverse 5′-TGAACGCCACTTGTCCCTCTA-3′. The primers crossed intron and exon junctions.

Data analysis. The data are expressed as the means ± SD. Comparison among more than two groups was performed by one-way ANOVA followed by the post hoc analysis (Bonferroni-Dunn test) to evaluate statistical significance between the two groups. All analyses were performed using StatView (SAS Institute, Cary, CA). Statistical significance was defined as $P < 0.05$.

RESULTS

Renal functional changes. Blood glucose and A1C were increased, and body weight was decreased in both diabetic rat groups compared with the control rat group. Systolic blood pressure remained in the normal range in the three groups, and no difference was found during the study period. The urine volume, Ccr, and albuminuria were increased in diabetic rats without olmesartan. However, treatment with olmesartan for 20 weeks significantly decreased Ccr and albuminuria, as shown in Table 1. As seen in STZ-induced diabetic rats, blood glucose and A1C were increased in *db/db* mice compared with db/m mice, while body weight was increased only in *db/db* mice (Table 2).

Expression of glomerular Smad1 and Col4 was increased at 4 weeks in diabetic rats. First, we examined whether mesangial expansion can be observed in an early

phase of diabetic nephropathy. We divided 14 diabetic rats into two groups according to the median of 1.74 ng/mg creatinine. Although we found no significant glomerular lesions by light microscopic analysis at 4 weeks in diabetic rats, glomerular expression of Smad1 and Col4 was already increased in some diabetic rats at this stage (Fig. 2). Because the level of urinary Smad1 was widely distributed, we divided diabetic rats into two groups: one is a group showing higher levels of urinary Smad1 at 4 weeks above the median and the other is a group showing lower levels of urinary Smad1 less than the median. As expected, glomerular Smad1 was significantly increased in the group with higher urinary Smad1 than in the group with lower urinary Smad1 (Fig. 3A and B). In these two groups, we measured mRNA levels of collagen α1(IV) obtained from the glomeruli by real-time PCR along with glomerular collagen α1(IV)–positive area by immunohistochemistry. Collagen α1(IV) mRNA and col4-positive areas were higher in diabetic rats with higher urinary Smad1 (Fig. 3C and D). Next, we checked the correlation between glomerular expression of Col4 and urinary Smad1. As shown in Fig. 3E, there was a statistically significant correlation between them ($r = 0.77$, $P = 0.001$).

Urinary Smad1 in an early phase of diabetes correlates with later onset of mesangial matrix expansion.
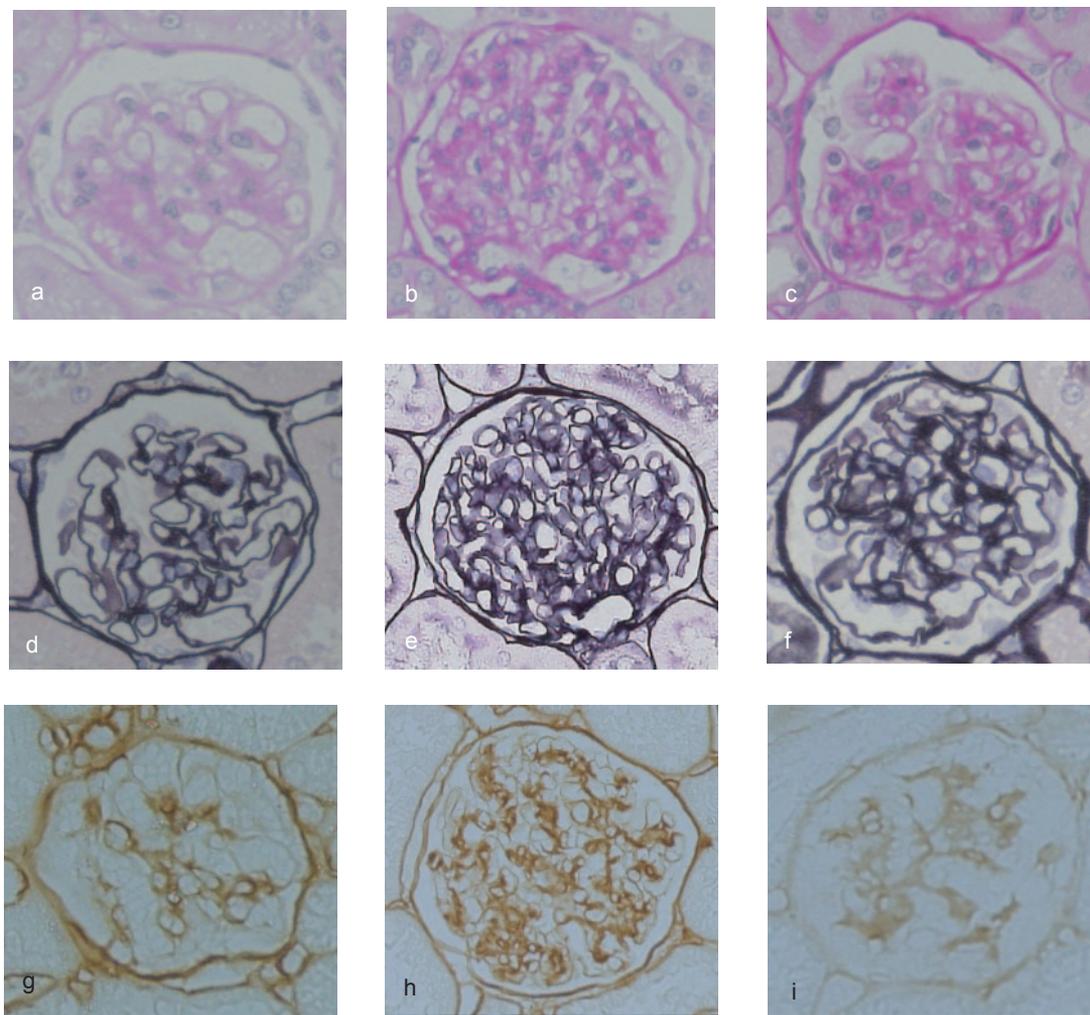
FIG. 6. Olmesartan ameliorates mesangial matrix expansion. Representative light microscopic appearance of glomeruli (PAS and PASM staining) and immunohistochemistry of Col4 for control rats (*a*, *d*, and *g*), diabetic rats (*b*, *e*, and *h*), and diabetic rats with olmesartan (*c*, *f*, and *i*). *a* and *c*: PAS staining. *d* and *f*: PASM staining. *g–i*: Col4. The original magnification was ×400.

We previously developed ELISAs to measure the concentration of urinary Smad1 (online appendix Fig. 1) (15,20). We reported that the glomerular expression of Smad1 is significantly increased and that urinary Smad1 is closely related to mesangial matrix expansion in diabetic rats at 24 weeks after STZ injection. Therefore, we examined whether this is also the case at 4 weeks after STZ injection. At the same time, we examined the relationship of mesangial matrix expansion with albuminuria and urinary excretion of Col4. As expected, urinary Smad1 at 4 weeks was nicely correlated with mesangial matrix expansion at 24 weeks in diabetic rats ($r = 0.70$, $P < 0.001$) (Fig. 4A), while urinary excretion of Smad1 was hardly detected in control rats (data not shown). We also found that albuminuria was correlated with mesangial matrix expansion, although its association was weaker than Smad1 ($r = 0.31$, $P = 0.043$) (Fig. 4B). We found no correlation between urinary excretion of Col4 and mesangial matrix expansion ($r = 0.01$, $P = 0.523$) (Fig. 4C). Moreover, there was no association between urinary Smad1 and albuminuria ($r = 0.04$, $P = 0.812$) (Fig. 4D). Also, there was no correlation of urinary Smad1 with blood pressure or serum glucose ($r = 0.13$, $P = 0.434$) (Fig. 4E) ($r = 0.24$, $P = 0.126$) (Fig. 4F). We obtained similar results when we analyzed urine samples at 24 weeks (data not shown).

Next, we examined whether urinary Smad1 in an early phase of diabetic nephropathy is correlated with later onset of mesangial matrix expansion in another diabetic model, *db/db* mice. At 18 weeks of age, mesangial matrix was significantly expanded in *db/db* mice (Fig. 5A), and there was a significant correlation between urinary Smad1 at 6 weeks and mesangial expansion ($r = 0.62$, $P = 0.033$) (Fig. 5B). As in STZ-induced diabetic rats, the association between albuminuria and mesangial matrix expansion was less prominent ($r = 0.55$, $P = 0.09$; Fig. 5C).

Olmesartan inhibits mesangial matrix expansion and decreases urinary excretion of Smad1 in diabetic rats. We previously reported that olmesartan ameliorates the development of diabetic nephropathy through the inhibition of Src and Smad1 (5). Therefore, in this study we investigated whether amelioration of morphological changes and mesangial matrix expansion can be correlated with the change of urinary Smad1 by olmesartan treatment in STZ-induced diabetic rats. Figure 6 shows a representative light microscopic picture and immunohistochemistry of Col4 in each group. Untreated diabetic rats showed an increase of mesangial matrix and mesangial matrix fraction compared with control rats, and this increase was diminished by olmesartan treatment. In untreated diabetic rats, the glomerular expression of
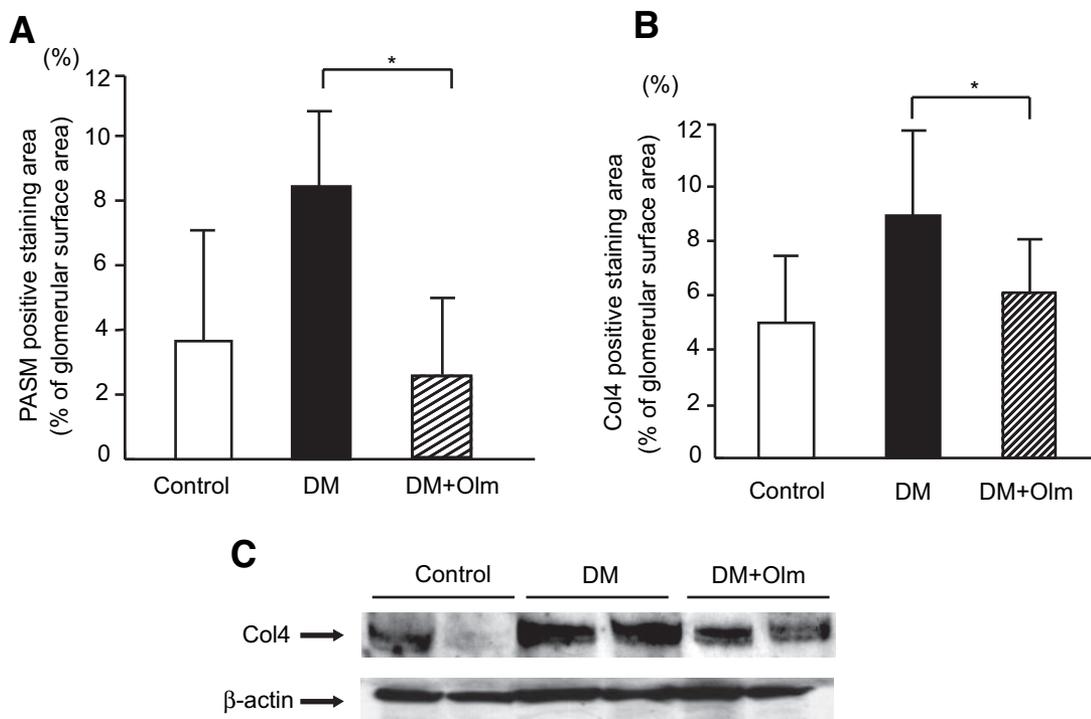
FIG. 7. Olmesartan ameliorates mesangial matrix expansion. *A*: Morphometric analysis of PASM positive staining area. *P < 0.05. *B*: Morphometric analysis of the glomerular expression of Col4. *P < 0.05. *C*: Immunoblots for Col4 in control and diabetic rats at 24 weeks are shown. Proteins (20 μg) obtained from each glomerular lysates were loaded. Each lane represents a representative Western blot for the glomerular lysates from each rat. Control, control rat; DM, STZ-induced diabetic rat with vehicle; DM+Olm, STZ-induced diabetic rats treated with olmesartan.

Col4 was significantly increased compared with that of control rats, while olmesartan treatment inhibited the glomerular expression of Col4 induced by diabetes (Fig. 7*B* and *C*).

We next examined the effect of olmesartan on urinary Smad1 in diabetic rats. In control rats, there was almost no excretion of Smad1. However, in untreated diabetic rats, urinary Smad1 was markedly increased along with an increase of glomerular Smad1 (data not shown). We found no significant change in urinary Smad1 between 4 and 24 weeks (3.3 ± 3.8 vs. 4.4 ± 7.7 ng/mg creatinine, $P = 0.383$) (Fig. 8*A*). In contrast, urinary Smad1 was dramatically decreased at 24 weeks compared with at 4 weeks in diabetic rats by olmesartan treatment (3.9 ± 2.9 vs. 0.3 ± 0.3 ng/mg creatinine, $P = 0.007$) (Fig. 8*B*), and its decrease was correlated with the morphological change of mesangial expansion in the early phase of diabetic nephropathy.

DISCUSSION

In this study, we have shown that urinary Smad1 in an early phase of diabetes can predict the future development of mesangial glomerular expansion in two rodent diabetic models and that the correlation of urinary Smad1 with glomerular expansion is better than that with albuminuria or urinary excretion of Col4. We have also shown that olmesartan treatment consistently ameliorated the morphological change in rat diabetic nephropathy as well as urinary excretion of Smad1. Thus, our data strongly indicate that urinary Smad1 could be a novel diagnostic marker to predict not only morphological changes but also the effect of ARB treatment on mesangial matrix expansion in diabetic nephropathy.

A number of clinical studies have shown a relationship between the extent of proteinuria or albuminuria and the

rates of progression of renal disease and complications (21,22). It is generally accepted that albuminuria can predict the later progression to overt proteinuria, and it is a useful diagnostic marker for predicting future overt nephropathy. However, as mentioned above, the sensitivity and specificity of albuminuria are not so high. Therefore, it is not always reliable. Indeed, in this study, we showed that albuminuria was associated with mesangial matrix expansion, but its association was weaker than that of urinary Smad1. Moreover, we found no association between urinary Smad1 and albuminuria. Thus, albuminuria may not be sufficient as a diagnostic marker to predict the future morphological changes. However, albuminuria is an effective marker to identify subjects who are at increased risk for metabolic syndrome, especially for cardiovascular events (23), because recent studies (24,25) show that albuminuria is a component of metabolic syndrome and may represent a marker of cardiovascular disease associated with insulin resistance and endothelial dysfunction. Therefore, albuminuria may be sustained by the inflammatory status that accompanies macrovascular disease (26). However, its ability to predict mesangial matrix expansion of diabetic nephropathy is weaker than urinary Smad1. Therefore, we propose that albuminuria can be used together with urinary Smad1 to predict diabetic nephropathy more accurately. In this study, although there were no significant glomerular lesions by light microscopic analysis at 4 weeks in diabetic rats, expression of glomerular Col4 was already increased at this stage. Further, urinary Smad1 could be detected at this early time point. Induction of Col4 transcription could be induced by Smad1 at this stage.

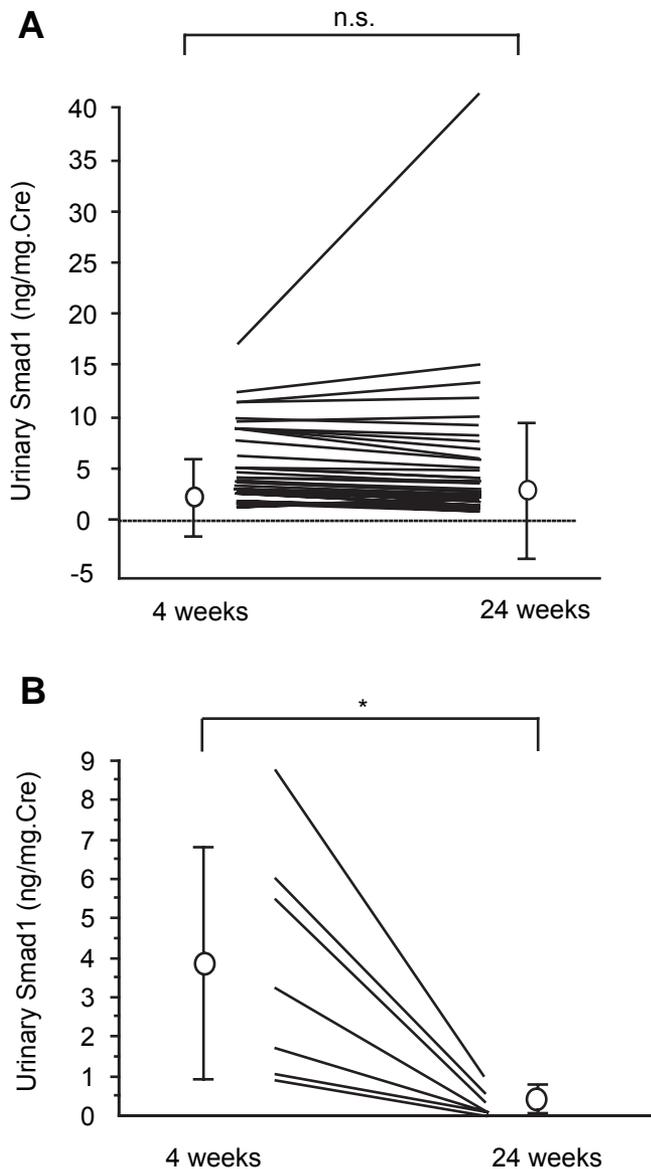Several cytokines, such as transforming growth factor-β or connective tissue growth factor, can be detected in the

FIG. 8. Urinary Smad1 was dramatically decreased by olmesartan in diabetic rats. *A*: There was no difference in untreated rats between at 4 weeks and at 24 weeks (3.3 ± 3.8 vs. 4.4 ± 7.7 ng/mg creatinine; *P* = 0.383 vs. 4 weeks). *B*: Olmesartan dramatically decreased urinary Smad1 (3.9 ± 2.9 vs. 0.3 ± 0.3 ng/mg creatinine; *P* < 0.05 vs. 4 weeks). The data are expressed as means ± SD. Note: The figures for this article can be found in the online appendix using the link entitled "Figures" (Available at http://dx.doi.org/10.2337/db07-1726).

morphological changes and these cytokines. In this study, we clearly showed that olmesartan treatment significantly ameliorated mesangial matrix expansion in diabetic rats, which was perfectly consistent with a decrease of urinary Smad1. Therefore, we proposed that urinary excretion of Smad1 would be a better diagnostic marker because it nicely reflects morphological changes by olmesartan in diabetic nephropathy.

The observed reduction of urinary Smad1 by olmesartan may reflect a specific inhibition of Smad1 expression because our previous study demonstrated that the expression of glomerular Smad1 is inhibited by olmesartan and that the Src/Smad1 pathway is inhibited by olmesartan (5). We found no relationship between blood pressure and urinary Smad1. Moreover, the reduction in urinary Smad1 cannot be explained by changes in blood glucose or A1C, since both variables are not different between diabetic rats with olmesartan and untreated diabetic rats. In this study, we have demonstrated an additional, blood pressure–independent reduction in urinary Smad1 by ARB therapy. Therefore, further study is necessary to clarify this mechanism. Clinical studies are underway to investigate the concept that urinary Smad1 could be an early predictor in diabetic patients.

It is well known that ARBs have renoprotection beyond blood pressure lowering (33) and that its renoprodective effect is dose dependent (34). We detected a significant decrease of urinary Smad1 in the 2 mg/kg group; in other words, we could hardly detect urinary Smad1 as in control rats (below the low standard), along with a significant decrease in blood pressure (2 mg/kg: 77.3 ± 7.6 mmHg, 0.6 mg/kg: 108 ± 6.1 mmHg). These findings would reflect dose dependence of the olmesartan effect.

We have shown that distinct glomerular hypertrophy was observed 12 weeks after STZ injection, which was ameliorated by warfarin treatment (18). In the warfarin treatment group, as with ARB treatment, urinary Smad1 was decreased and glomerular expression of Smad1 was inhibited (diabetes without warfarin: 3.1 ± 1.2 ng/mg creatinine vs. diabetes with warfarin 1.6 ± 0.6 ng/mg creatinine; *P* = 0.05) (online appendix Fig. 2).

One question may arise about the source of urinary Smad1. We assume that urinary Smad1 comes from mesangial cells because both Smad1 and phospho-Smad1 were dominantly detected in mesangial cells by immunohistochemistry. However, Smad1 is also found in endothelial cells (35), and urinary podocyte excretion is shown in diabetic nephropathy (36). These data suggest that urinary Smad1 might also derive from podocytes or endothelial cells. There is another possibility that Smad1 in serum is excreted in the urine. However, we could hardly detect Smad1 using our ELISA system. Therefore, this possibility can be excluded. Further investigation is necessary to determine the mechanism and source of urinary Smad1.

Recently, it has been reported that Smad5, not Smad1, is the preferred R-Smad for bone morphogetic protein 7 (BMP7) in proximal tubular cells, mesangial cells, and podocytes and that BMP7 has a renoprotective effect (37–39). Therefore, the role of BMP and Smads in diabetic nephropathy should be further addressed in future studies. Whether Smad1 is injurious or protective for diabetic nephropathy needs to be examined by overexpression or knockout of Smad1 in mesangial cells. A study is now underway to determine the specific role of Smad1 in

urine of diabetic patients, and several groups found the relationship between those urinary cytokines and albuminuria or glomerular filtration rate. However, these markers generally increase in the relatively late phase of the disease, and interpretation of these markers might be difficult because of their high ubiquity and multiple forms.

ARB treatment for diabetic nephropathy lately has been paid considerable attention by a number of studies (27–29) that show the effect of ARBs for diabetic nephropathy. However, the only way to detect the effect of ARBs on mesangial matrix expansion in the early phase of diabetic nephropathy has been renal biopsy. In addition, ARB treatment is known to reduce urinary transforming growth factor-β, connective tissue growth factor, or Col4 in diabetic renal disease (3,30–32). However, no report demonstrates the relationship of

diabetic nephropathy by making transgenic and knockout mice.

It is intriguing to address the role of Smad1 in nondiabetic ECM accumulation models. Therefore, we examined whether urinary Smad1 is also detected in a nondiabetic ECM accumulation model. However, we could not detect urinary Smad1 in rats with Thy1 nephritis (data not shown). Although both diabetic nephropathy and Thy1 nephritis accumulate ECM, why urinary Smad1 was not detected in Thy1 nephritis is still unclear. Further investigation is necessary to elucidate this mechanism.

In this study, we have clearly shown that urinary Smad1 in a very early stage of diabetes is significantly correlated with the later development of mesangial matrix expansion followed by glomerulosclerosis and that olmesartan treatment significantly ameliorated mesangial matrix expansion, perfectly consistent with a decrease of urinary Smad1. These data indicate that urinary Smad1 could be a novel predictor for future morphological damages and can be used to monitor the effect of ARBs in diabetic nephropathy.

REFERENCES

1. Ziyadeh FN: The extracellular matrix in diabetic nephropathy. *Am J Kidney Dis* 22:736–744, 1993
2. Lewis EJ, Hunsicker LG, Clarke WR, Berl T, Pohl MA, Lewis JB, Ritz E, Atkins RC, Rohde R, Raz I: Renoprotective effect of the angiotensin-receptor antagonist irbesartan in patients with nephropathy due to type 2 diabetes. *N Engl J Med* 345:851–860, 2001
3. Liao J, Kobayashi M, Kanamuru Y, Nakamura S, Makita Y, Funabiki K, Horikoshi S, Tomino Y: Effects of candesartan, an angiotensin II type 1 receptor blocker, on diabetic nephropathy in KK/Ta mice. *J Nephrol* 16:841–849, 2003
4. Abe H, Matsubara T, Iehara N, Nagai K, Takahashi T, Arai H, Kita T, Doi T: Type IV collagen is transcriptionally regulated by Smad1 under advanced glycation end product (AGE) stimulation. *J Biol Chem* 279:14201–14206, 2004
5. Mima A, Matsubara T, Arai H, Abe H, Nagai K, Kanamori H, Sumi E, Takahashi T, Iehara N, Fukatsu A, Kita T, Doi T: Angiotensin II-dependent Src and Smad1 signaling pathway is crucial for the development of diabetic nephropathy. *Lab Invest* 86:927–939, 2006
6. Keen H, Chlouverakis C, Fuller J, Jarrett RJ: The consomitants of raised blood sugar: studies in newly-detected hyperglycaemics: II. urinary albumin excretion, blood pressure and their relation to blood sugar levels. *Guys Hosp Rep* 118:247–254, 1969
7. Bilous RW, Mauer SM, Sutherland DE, Najarian JS, Goetz FC, Steffes MW: The effects of pancreas transplantation on the glomerular structure of renal allografts in patients with insulin-dependent diabetes. *N Engl J Med* 321:80–85, 1989
8. Mogensen CE: Long-term antihypertensive treatment inhibiting progression of diabetic nephropathy. *Br Med J (Clin Res Ed)* 285:685–688, 1982
9. Chavers BM, Bilous RW, Ellis EN, Steffes MW, Mauer SM: Glomerular lesions and urinary albumin excretion in type I diabetes without overt proteinuria. *N Engl J Med* 320:966–970, 1989
10. Fioretto P, Steffes MW, Mauer M: Glomerular structure in nonproteinuric IDDM patients with various levels of albuminuria. *Diabetes* 43:1358–1364, 1994
11. Ellis D, Forrest KY, Erbey J, Orchard TJ: Urinary measurement of transforming growth factor-beta and type IV collagen as new markers of renal injury: application in diabetic nephropathy. *Clin Chem* 44:950–956, 1998
12. Riser BL, Cortes P, DeNichilo M, Deshmukh PV, Chahal PS, Mohammed AK, Yee J, Kahkonen D: Urinary CCN2 (CTGF) as a possible predictor of diabetic nephropathy: preliminary report. *Kidney Int* 64:451–458, 2003
13. Makibayashi K, Tatematsu M, Hirata M, Fukushima N, Kusano K, Ohashi S, Abe H, Kuze K, Fukatsu A, Kita T, Doi T: A vitamin D analog ameliorates glomerular injury on rat glomerulonephritis. *Am J Pathol* 158:1733–1741, 2001
14. Myint KM, Yamamoto Y, Doi T, Kato I, Harashima A, Yonekura H, Watanabe T, Shinohara H, Takeuchi M, Tsuneyama K, Hashimoto N, Asano M, Takasawa S, Okamoto H, Yamamoto H: RAGE control of diabetic nephropathy in a mouse model: effects of RAGE gene disruption and administration of low-molecular weight heparin. *Diabetes* 55:2510–2522, 2006
15. Matsubara T, Abe H, Arai H, Nagai K, Mima A, Kanamori H, Sumi E, Takahashi T, Matsuura H, Iehara N, Fukatsu A, Kita T, Doi T: Expression of Smad1 is directly associated with mesangial matrix expansion in rat diabetic nephropathy. *Lab Invest* 86:357–368, 2006
16. Nagai K, Arai H, Yanagita M, Matsubara T, Kanamori H, Nakano T, Iehara N, Fukatsu A, Kita T, Doi T: Growth arrest-specific gene 6 is involved in glomerular hypertrophy in the early stage of diabetic nephropathy. *J Biol Chem* 278:18229–18234, 2003
17. Yamamoto Y, Kato I, Doi T, Yonekura H, Ohashi S, Takeuchi M, Watanabe T, Yamagishi S, Sakurai S, Takasawa S, Okamoto H, Yamamoto H: Development and prevention of advanced diabetic nephropathy in RAGE-overexpressing mice. *J Clin Invest* 108:261–268, 2001
18. Nagai K, Matsubara T, Mima A, Sumi E, Kanamori H, Iehara N, Fukatsu A, Yanagita M, Nakano T, Ishimoto Y, Kita T, Doi T, Arai H: Gas6 induces Akt/mTOR-mediated mesangial hypertrophy in diabetic nephropathy. *Kidney Int* 68:552–561, 2005
19. Stockelman MG, Lorenz JN, Smith FN, Boivin GP, Sahota A, Tischfield JA, Stambrook PJ: Chronic renal failure in a mouse model of human adenine phosphoribosyltransferase deficiency. *Am J Physiol* 275:F154–F163, 1998
20. Hayashi Y, Matsuda R, Maitani T, Imai K, Nishimura W, Ito K, Maeda M: Precision, limit of detection and range of quantitation in competitive ELISA. *Anal Chem* 76:1295–1301, 2004
21. Mogensen CE, Chachati A, Christensen CK, Close CF, Deckert T, Hommel E, Kastrup J, Lefebvre P, Mathiesen ER, Feldt-Rasmussen B, et al.: Microalbuminuria: an early marker of renal involvement in diabetes. *Uremia Invest* 9:85–95, 1985
22. Dinneen SF, Gerstein HC: The association of microalbuminuria and mortality in non-insulin-dependent diabetes mellitus: a systematic overview of the literature. *Arch Intern Med* 157:1413–1418, 1997
23. Isomaa B, Almgren P, Tuomi T, Forsen B, Lahti K, Nissen M, Taskinen MR, Groop L: Cardiovascular morbidity and mortality associated with the metabolic syndrome. *Diabetes Care* 24:683–689, 2001
24. Adler AI, Stevens RJ, Manley SE, Bilous RW, Cull CA, Holman RR: Development and progression of nephropathy in type 2 diabetes: the United Kingdom Prospective Diabetes Study (UKPDS 64). *Kidney Int* 63:225–232, 2003
25. Chen J, Muntner P, Hamm LL, Jones DW, Batuman V, Fonseca V, Whelton PK, He J: The metabolic syndrome and chronic kidney disease in U.S. adults. *Ann Intern Med* 140:167–174, 2004
26. Henareh L, Jogestrand T, Agewall S: Microalbuminuria in patients with previous myocardial infarction. *Kidney Int* 69:178–183, 2006
27. Lewis EJ, Hunsicker LG, Bain RP, Rohde RD: The effect of angiotensin-converting-enzyme inhibition on diabetic nephropathy: the Collaborative Study Group. *N Engl J Med* 329:1456–1462, 1993
28. Brenner BM, Cooper ME, de Zeeuw D, Keane WF, Mitch WE, Parving HH, Remuzzi G, Snapinn SM, Zhang Z, Shahinfar S: Effects of losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy. *N Engl J Med* 345:861–869, 2001
29. 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
30. Esmatjes E, Flores L, Inigo P, Lario S, Ruilope LM, Campistol JM: Effect of losartan on TGF-beta1 and urinary albumin excretion in patients with type 2 diabetes mellitus and microalbuminuria. *Nephrol Dial Transplant* 16 (Suppl. 1):90–93, 2001
31. Houlihan CA, Akdeniz A, Tsalamandris C, Cooper ME, Jerums G, Gilbert RE: Urinary transforming growth factor-beta excretion in patients with hypertension, type 2 diabetes, and elevated albumin excretion rate: effects of angiotensin receptor blockade and sodium restriction. *Diabetes Care* 25:1072–1077, 2002
32. Ogawa S, Mori T, Nako K, Kato T, Takeuchi K, Ito S: Angiotensin II type 1 receptor blockers reduce urinary oxidative stress markers in hypertensive diabetic nephropathy. *Hypertension* 47:699–705, 2006

33. Izuhara Y, Nangaku M, Inagi R, Tominaga N, Aizawa T, Kurokawa K, van Ypersele de Strihou C, Miyata T: Renoprotective properties of angiotensin receptor blockers beyond blood pressure lowering. *J Am Soc Nephrol* 16:3631–3641, 2005

34. Fujihara CK, Velho M, Malheiros DM, Zatz R: An extremely high dose of losartan affords superior renoprotection in the remnant model. *Kidney Int* 67:1913–1924, 2005

35. Upton PD, Long L, Trembath RC, Morrell NW: Functional characterization of BMP binding sites and Smad1/5 activation in human vascular cells. *Mol Pharmacol* 73:539–552, 2007

36. Nakamura T, Uchiyama C, Suzuki S: Urinary excretion of podocytes in patients with diabetic nephropathy. *Nephrol Dial Transplant* 15:1379–1383, 2000

37. Wang S, Hirshberg R: Bone morphogenetic protein-7 signals opposing transforming growth factor β in mesangial cells. *J Biol Chem* 279:23200–23206, 2004

38. Mitu GM, Wang S, Hirschberg R: BMP7 is a podocyte survival factor and rescues podocytes from diabetic injury. *Am J Renal Physiol* 293:1641–1648, 2007

39. Wang S, Lapage J, Hirscberg R: Loss of tubular bone morphogenic protein-7 in diabetic nephropathy. *J Am Soc Nephrol* 12:2392–2399, 2001