Lovastatin Inhibits Transforming Growth Factor-β1 Expression in Diabetic Rat Glomeruli and Cultured Rat Mesangial Cells

SUNG IL KIM, DONG CHEOL HAN, and HI BAHL LEE
Hyonam Kidney Laboratory, Soon Chun Hyang University, Seoul, Korea.

Abstract. Diabetic nephropathy is a leading cause of end-stage renal disease and is characterized by excessive deposition of extracellular matrix (ECM) proteins in the glomeruli. Transforming growth factor-β (TGF-β) is the major mediator of excessive accumulation of ECM proteins in diabetic nephropathy through upregulation of genes encoding ECM proteins as well as downregulation of genes for ECM-degrading enzymes. It has been shown that lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl CoA reductase, delays the onset and progression of different models of experimental nephropathy. To evaluate the effect of lovastatin on the development and progression of diabetic nephropathy, streptozotocin-induced diabetic rats were studied for 12 mo. In untreated diabetic rats, there were significant increases in blood glucose, urine albumin excretion, kidney weight, glomerular volume, and TGF-β1 mRNA expression in the glomeruli compared with normal control rats treated with citrate buffer only. Treatment with lovastatin in diabetic rats significantly suppressed the increase in urine albumin excretion, kidney weight, glomerular volume, and TGF-β1 mRNA expression despite high blood glucose levels. To elucidate the mechanisms of the renal effects of lovastatin, rat mesangial cells were cultured under control (5.5 mM) or high (30 mM) glucose with lovastatin alone, mevalonate alone, or with both. Under high glucose, TGF-β1 and fibronectin mRNA and proteins were upregulated. These high glucose-induced changes were suppressed by lovastatin (10 μM) and nearly completely restored by mevalonate (100 μM). These results suggest that lovastatin has a direct cellular effect independent of a cholesterol-lowering effect and delays the onset and progression of diabetic nephropathy, at least in part, through suppression of glomerular expression of TGF-β1.

Excessive deposition of extracellular matrix (ECM) proteins in the glomeruli and subsequent mesangial expansion are the main structural alterations in diabetic nephropathy (1). Both *in vitro* and *in vivo* studies have shown that high glucose stimulates expression of genes encoding ECM proteins in tubular epithelial cells as well as mesangial cells (2–5). These effects are largely mediated by upregulation of transforming growth factor-β (TGF-β) expression and/or activation of TGF-β (5–7). TGF-β induces accumulation of ECM proteins by both stimulating synthesis of ECM proteins (8) and decreasing activity of ECM-degrading enzymes (8,9). For these reasons, TGF-β has been proposed as the therapeutic target in diabetic nephropathy (10).

It has been shown that the inhibition of 3-hydroxy-3-methylglutaryl CoA (HMG CoA) reductase with lovastatin is associated with amelioration of albuminuria and glomerulosclerosis in 5/6 nephrectomized rats (11,12), obese Zucker rats (13), and streptozotocin-induced diabetic uninephrectomized rats (14). Because lovastatin also reduces serum cholesterol levels in these experimental models, the beneficial effect of lovastatin has generally been attributed to a decrease in circulating levels of cholesterol. There is evidence, however, that the effect of lovastatin on glomerular structure and function may result, at least in part, from a direct action at the cellular level. Lovastatin has been shown to inhibit mesangial cell proliferation (15) and induce apoptosis in cultured mesangial cells (16). Lovastatin has been shown to inhibit NF-κB activation (17) and monocyte chemoattractant protein-1 production (18), and modulate the plasminogen activator-plasmin system (19). We have demonstrated that lovastatin effectively suppresses TGF-β1 mRNA expression in diabetic rat glomeruli 2 d after induction of diabetes (20). These studies suggest that the beneficial effect of lovastatin on diabetic nephropathy may be due to its direct effect on TGF-β and ECM synthesis. However, there is no long-term study showing that lovastatin prevents the renal manifestations of diabetic nephropathy or suppresses TGF-β expression and accumulation of ECM in diabetic glomeruli. Therefore, we explored the effects of lovastatin on the expression of TGF-β1 in both streptozotocin-induced diabetic rat glomeruli and in rat mesangial cells cultured under high glucose concentration and assessed the effect of lovastatin on the expression of fibronectin in these cells.

Materials and Methods

Experimental Animals

Male Sprague Dawley rats weighing 200 to 250 g were used. Diabetes was induced by a single tail vein injection of 65 mg/kg body

Received April 6, 1999. Accepted June 25, 1999.
Correspondence to Dr. Hi Bahl Lee, Hyonam Kidney Laboratory, Soon Chun Hyang University, 657 Hannam Dong, Yongsan Ku, Seoul 140-743, Korea. Phone: +82 2 709 9171, +82 2 792 6657; Fax: +82 2 792 5812; E-mail: hblee@korea.com

1046-6673/1101-0080
Journal of the American Society of Nephrology

wt of streptozotocin (Sigma Chemical Co., St. Louis, MO) dissolved in citrate buffer (pH 4.5) immediately before injection. Diabetes mellitus was confirmed by measuring tail venous blood glucose levels using a reflectance meter (Reflolux S, Boehringer Mannheim, Mannheim, Germany), and rats with blood glucose level <250 mg/dl were excluded from the experiment. Diabetic animals were given small doses of insulin as needed to maintain blood glucose levels at a medium range (400 to 600 mg/dl). Three different groups of animals were prepared: normal age-matched control rats (NC) receiving citrate buffer (pH 4.5) only, untreated diabetic rats (DD), and diabetic rats treated with lovastatin (gift from Choong Wae Pharmaceutical Co., Seoul, Korea) (DL). Lovastatin was dissolved in a mix of propylene glycol and ethanol (vol/vol) to give a concentration of 8 mg/ml and was injected daily subcutaneously with a dose of 4 mg/kg of body weight beginning 24 h after induction of diabetes. All rats had free access to water and standard rat chow (Samyang Food and Feed Co., Seoul, Korea).

Serum Cholesterol and Creatinine and Urine Albumin Excretion

Serum cholesterol was measured by an enzyme-linked (cholesterol hydrolase/cholesterol oxidase/peroxidase) colorimetric method using Cholesterol-E kit (Yeong Dong Pharmaceutical Co., Kyunggi, Korea). Serum creatinine was measured by modified Jaffe method using Creatinine-E kit (Yeong Dong Pharmaceutical Co.). Twenty-four-hour urine samples were obtained from animals in metabolic cages with access to drinking water only. Urine albumin was measured by a quantitative reaction with bromcresol green.

Mean Glomerular Volume

Glomerular volume (V_G) was calculated according to the method of Weibel (21) as described previously (22). In brief, surface areas of 75 to 100 glomeruli selected randomly were measured with Image-pro (version 1.2; Media Cybernetics, Silver Spring, MD) software program on periodic acid-Schiff-stained tissue sections, and V_G was calculated using the equation:

$$V_G = \text{Area}^{1.5} \times 1.38/1.01,$$

where V_G is glomerular volume, 1.38 represents the shape coefficient, and 1.01 is the size distribution coefficient. The validity of this method was tested, and V_G determined by this method was found to correlate with that from the Cavalieri method ($r = 0.68$, $P < 0.05$) and somewhat better with maximal profile area method ($r = 0.83$, $P < 0.0001$). A strong correlation was found between volumes determined by Cavalieri and the maximal profile area technique ($r = 0.93$; $P < 0.01$) (23).

Rat Glomeruli Isolation and Mesangial Cell Culture

Rat glomeruli were isolated from 8-wk-old male rat kidney according to the sieving method (24). Mesangial cells were obtained from the isolated glomeruli after treatment with collagenase and identified by typical stellate morphology, positive staining for the intermediate filaments, desmin, and vimentin (24). Rat mesangial cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY) containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.5 μg/ml fungizone as described previously (5). As the cells reached 80% confluence in 100-mm culture dishes, cells were incubated in serum-free media for 2 d to arrest and synchronize cell growth. Cells were then treated with serum-free media containing either control (5.5 mM) or high (30 mM) glucose. At the beginning of glucose treatment, lovastatin (10 μM) or mevalonate (100 μM) (Sigma Chemical Co.) was added to the culture alone or together, and the cells were incubated for 24 to 48 h. After incubation, culture supernatant was centrifuged to remove cell debris and was used in Western blot analysis for secreted TGF-β and fibronectin proteins. Total RNA was prepared from the cultured cells for Northern analysis of TGF-β1 and fibronectin mRNA.

Preparation of Lovastatin and Mevalonate for Use in Cell Culture

Inactive lactone forms of lovastatin and mevalonate were converted to their active forms as described before (15,25). In brief, 40 mg of the inactive lovastatin was dissolved in 1 ml of absolute ethanol, and 0.1 M NaOH was added, heated at 50°C for 2 h, and neutralized with 0.1 M HCl to pH 7.2. The final volume was adjusted to 20 ml with distilled water. Mevalonate (32.5 mg) was dissolved in 2 ml of absolute ethanol, 23 ml of RPMI 1640 (Life Technologies) was added, and pH was adjusted to 7.4. The prepared lovastatin and mevalonate were stored at −20°C until use.

Isolation of Total RNA and Northern Blot Analysis

Total RNA from the isolated glomeruli was prepared using TRI-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's description after homogenization with motor-driven pellet pestle (Sigma Chemical Co.). From the cultured mesangial cells, total RNA was obtained by using RNeasy mini total RNA kits (Qiagen, Valencia, CA) according to the manufacturer's instructions. For Northern blot analysis, an equal amount of total RNA (approximately 15 to 20 μg) was applied on 1.0% agarose gel containing 2.2 M formaldehyde after denaturation. After gel electrophoresis, fractionated RNA was transferred onto nylon membranes and cross-linked by irradiation with ultraviolet light. Rat TGF-β1 cDNA (American Type Culture Collection, Rockville, MD) and partial cDNA for rat fibronectin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cloned after reverse transcription-PCR and confirmed by DNA sequencing were used for the respective DNA probes. Prehybridization was performed at 65°C for 2 h. Hybridization was carried out at 65°C for 16 h with the DNA probe labeled radioactively using 5 μCi of ^{32}P-α-dCTP (3000 Ci/mmol) and Ready-To-Go random DNA labeling kit (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). After hybridization, the nylon membrane was washed at 65°C for 15 min with 2× SSC (20× SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0) containing 0.5% sodium dodecyl sulfate (SDS) followed by washing with 0.5× SSC containing 0.1% SDS under the same conditions. At the final step, the membrane was washed with 0.1× SSC containing 0.1% SDS at 50°C for 10 min. After washing, air-dried membrane was autoradiographed using Kodak x-ray film with intensifying screen at −70°C for appropriate times. For the subsequent rehybridization with a different DNA probe, the blot was stripped at 80°C with stripping solution (0.1× SSC plus 0.5% SDS). Quantification of mRNA signals was carried out by densitometry using GS-700 imaging densitometer (Bio-Rad, Hercules, CA) and normalized with GAPDH mRNA signal.

Western Blot Analysis

TGF-β and fibronectin secreted from cultured mesangial cells were analyzed by Western blot. The respective supernatant containing an appropriate amount of protein was subjected to SDS-polyacrylamide gel electrophoresis (15% gel for TGF-β and 8% gel for fibronectin). After completion of electrophoresis, proteins were transferred onto nitrocellulose membrane (Hybond N, Amersham Pharmacia Biotech) in transfer buffer (50 mM Tris-HCl, pH 7.0, 380 mM glycine, and

20% methanol). The blots were blocked in 5% nonfat dry milk dissolved in phosphate-buffered saline with 0.1% Tween 20. The membranes were incubated for 1 h with polyclonal rabbit pan-specific anti-TGF-β antibody (R&D Systems, Minneapolis, MN) or rabbit anti-rat fibronectin antibody (Chemicon International, Temecula, CA). The secondary antibody was peroxidase-conjugated goat anti-rabbit IgG antibody (Cappel, Durham, NC). The luminescence detection of peroxidase was performed with the enhanced chemiluminescence system (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The relative amount of positive immunoreactive proteins was quantified with densitometric analysis using a GS-700 imaging densitometer (Bio-Rad).

Statistical Analyses

Results were expressed as mean ± SEM. ANOVA was used to assess the differences between multiple groups. If the F statistic was significant, the mean values obtained from each group were then compared by the Fisher least significant different method. A P value <0.05 was used to indicate a statistically significant difference.

Results

Effect of Lovastatin on Blood Glucose, Serum Cholesterol, and Creatinine, Body, and Kidney Weights in Diabetic Rats

Blood glucose levels of untreated and lovastatin-treated diabetic rats were significantly higher than those of normal control rats during the whole experimental period after induction of diabetes (Table 1). Concentrations of serum cholesterol in untreated diabetic rats were slightly higher than those in normal control rats and were significantly lower in diabetic rats treated with lovastatin than those in untreated diabetic rats at 6 and 12 mo (Table 1). Serum creatinine levels were not significantly different among the three groups during 12 mo after induction of diabetes (data not shown). Changes in body and kidney weights in the three groups of rats are shown in Table 2. Untreated diabetic rats had significantly lower body weight than control rats from 1 to 12 mo of diabetes. Lovastatin-treated rats also had lower body weight than control rats from 1 to 6 mo of diabetes but had weight similar to control rats at 12 mo. Kidney weight of untreated diabetic rats was significantly heavier compared with control rats at 1, 6, and 12 mo, and lovastatin effectively prevented the increase in kidney weight in diabetic rats.

Effect of Lovastatin on Urine Albumin Excretion and Mean Glomerular Volume in Diabetic Rats

Changes in 24-h urine albumin excretion (UAE) were monitored at 1, 3, 6, and 12 mo after induction of diabetes (Figure 1). A progressive increase in UAE with time was found in untreated diabetic rats. UAE in untreated diabetic rats was sevenfold higher than in control rats at 12 mo of diabetes. In contrast, UAE was significantly lower in lovastatin-treated rats than in untreated diabetic rats and was not significantly different from that in control rats at 6 and 12 mo. The changes in mean glomerular volume from the three experimental groups are illustrated in Figure 2. The mean glomerular volume of untreated diabetic rats was 37 and 47% larger than that of control rats at 6 and 12 mo of diabetes, respectively. Lovastatin effectively prevented the glomerular hypertrophy associated with diabetes, and the mean glomerular volume of lovastatin-treated rats was not different from that of control rats at 6 and 12 mo.

Effect of Lovastatin on TGF-β1 mRNA Expression in Diabetic Rat Glomeruli

Total RNA was obtained from the glomeruli at 4 d, 1 wk, 4 wk, and 12 wk after induction of diabetes, and Northern blot analysis was performed for TGF-β1 mRNA. There was a 1.3-fold (4 d) to 1.9-fold (12 wk) increase in glomerular TGF-β1 mRNA in untreated diabetic rats compared with control rats. Lovastatin effectively suppressed glomerular TGF-β1 mRNA expression in diabetic rats as shown in Figure 3. There

Table 1. Blood glucose and serum cholesterol levels in normal control and diabetic rats[a]

Group	Blood Glucose Level (mg/dl)					
	7 Days	14 Days	1 Month	3 Months	6 Months	12 Months
NC	168 ± 5 (12)	167 ± 13 (12)	189 ± 13 (12)	192 ± 22 (11)	201 ± 36 (11)	210 ± 57 (10)
DD	460 ± 15 (12)[b]	470 ± 10 (12)[b]	490 ± 15 (11)[b]	510 ± 21 (11)[b]	528 ± 29 (9)[b]	555 ± 41 (7)[b]
DL	472 ± 18 (12)[c]	477 ± 13 (12)[c]	502 ± 16 (10)[c]	505 ± 37 (10)[c]	532 ± 59 (9)[c]	540 ± 38 (7)[c]
	Serum Cholesterol (mg/dl)					
NC	62 ± 16 (12)	58 ± 1 (12)	62 ± 13 (12)	79 ± 6 (11)	58 ± 5 (11)	57 ± 3 (10)
DD	67 ± 5 (12)	58 ± 7 (12)	71 ± 2 (11)	67 ± 5 (11)	87 ± 16 (9)[b]	69 ± 5 (7)
DL	60 ± 4 (12)	55 ± 4 (12)	73 ± 4 (10)	57 ± 4 (10)	62 ± 10 (7)[d]	51 ± 5 (6)[d]

[a] Tail venous blood glucose level was measured using a reflectance meter (Reflolux S, Boehringer Mannheim, Mannheim, Germany). Serum cholesterol was measured by an enzyme-linked (cholesterol hydrolase/cholesterol oxidase/peroxidase) colorimetric method using Cholesterol-E kit (Yeong Dong Pharmaceutical Co., Kyunggi, Korea). Values are given as mean ± SEM, and the numbers in parentheses represent the number of animals examined. NC, age-matched normal control rats; DD, untreated diabetic rats; DL, diabetic rats treated with lovastatin.

[b] $P < 0.05$ DD *versus* NC.

[c] $P < 0.05$ DL *versus* NC.

[d] $P < 0.05$ DL *versus* DD.

Table 2. Body weight and kidney weight in normal control and diabetic rats[a]

Group	Body Weight (g)					
	7 Days	14 Days	1 Month	3 Months	6 Months	12 Months
NC	231 ± 13 (12)	263 ± 63 (12)	326 ± 13 (12)	410 ± 22 (11)	507 ± 36 (11)	557 ± 57 (10)
DD	213 ± 5 (12)	241 ± 10 (12)	281 ± 15 (11)[b]	394 ± 21 (11)[b]	430 ± 29 (9)[b]	497 ± 51 (7)[b]
DL	211 ± 18 (12)	232 ± 13 (12)	259 ± 16 (10)[c]	375 ± 37 (10)[c]	413 ± 59 (7)[c]	590 ± 38 (6)[d]
	Kidney Weight (g)					
NC	0.91 ± 0.06 (24)	0.94 ± 0.02 (24)	1.06 ± 0.04 (24)	1.47 ± 0.04 (22)	1.66 ± 0.13 (22)	1.82 ± 0.15 (20)
DD	1.08 ± 0.07 (24)	1.16 ± 0.17 (24)	1.23 ± 0.06 (22)[b]	1.53 ± 0.05 (22)	2.07 ± 0.07 (18)[b]	2.58 ± 0.15 (14)[b]
DL	0.93 ± 0.05 (24)	1.04 ± 0.17 (24)	1.19 ± 0.04 (20)	1.44 ± 0.06 (20)	1.72 ± 0.07 (14)[d]	1.77 ± 0.07 (12)[d]

[a] Values are given as mean ± SEM, and the numbers in parentheses represent the number of animals or kidneys examined. Abbreviations as in Table 1.
[b] $P < 0.05$ DD *versus* NC.
[c] $P < 0.05$ DL *versus* NC.
[d] $P < 0.05$ DL *versus* DD.

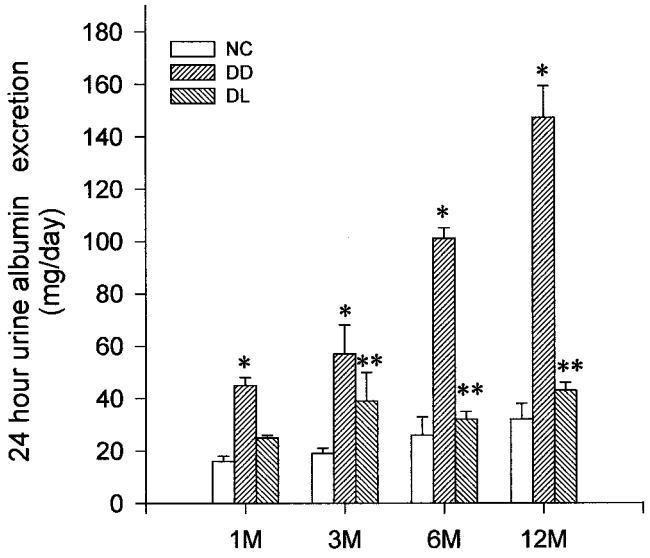

Figure 1. Effect of lovastatin on 24-h urine albumin excretion in control and diabetic rats. Twenty-four-hour urine samples were obtained from animals in metabolic cages with access to drinking water only. Ten animals in each group were examined at the indicated experimental period. Urine albumin was measured by a quantitative reaction with bromcresol green. Obtained values are represented as mean ± SEM. NC, age-matched normal control rats; DD, untreated diabetic rats; DL, diabetic rats treated with lovastatin. *$P < 0.05$ DD *versus* NC; **$P < 0.05$ DL *versus* DD.

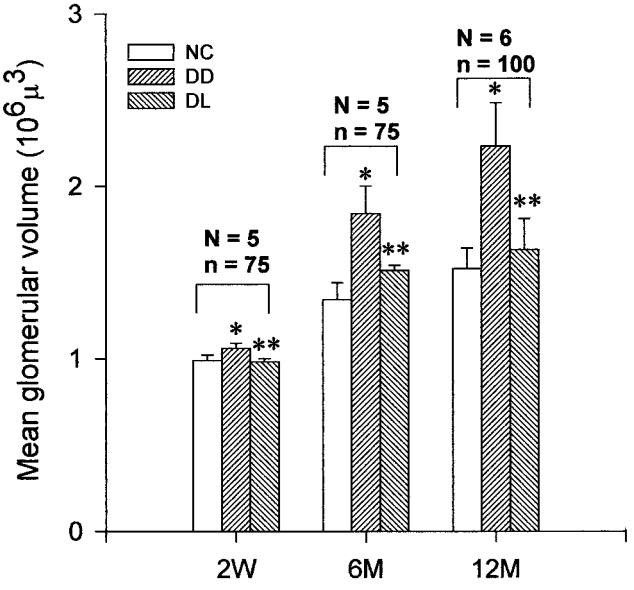

Figure 2. Changes in mean glomerular volume in control and diabetic rats. Surface areas of 75 to 100 glomeruli selected randomly were measured with the Image-pro (version 1.2; Media Cybernetic) software program on periodic acid-Schiff-stained tissue sections, and glomerular volume (V_G) was calculated from area measured by the method of Weibel (21):

$$V_G = \text{Area}^{1.5} \times 1.38/1.01,$$

where V_G is glomerular volume, 1.38 represents the shape coefficient, and 1.01 is the size distribution coefficient. Obtained values are presented as mean ± SEM, *n* is the number of glomeruli examined, and *N* represents the number of rats used in the morphometric study. Abbreviations as in Figure 1. *$P < 0.05$ DD *versus* NC; **$P < 0.05$ DL *versus* DD.

were significant differences in TGF-β1 mRNA expression between untreated and control and between untreated and lovastatin-treated diabetic rats in all periods ($P < 0.05$).

Effect of Lovastatin on TGF-β1 mRNA Expression and Protein Synthesis in Cultured Rat Mesangial Cells

To confirm the effect of lovastatin on TGF-β1 mRNA expression and protein synthesis, rat mesangial cells were cultured under control (5.5 mM) or high (30 mM) glucose concentration with or without lovastatin (10 μM). In addition, activated mevalonate (100 μM) was added in the culture alone or together with lovastatin (Figure 4). High glucose upregulated TGF-β1 mRNA expression in mesangial cells, and this upregulation was effectively blocked by lovastatin. Lovastatin

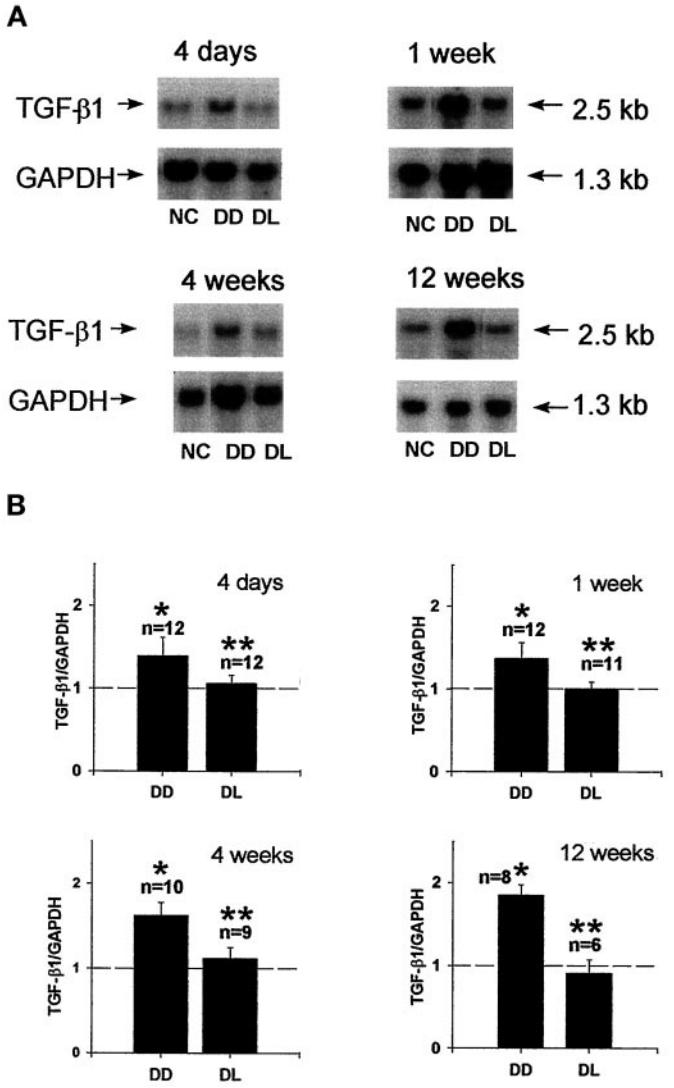

Figure 3. Effect of lovastatin on glomerular transforming growth factor-β1 (TGF-β1) mRNA expression in control and diabetic rats. Total RNA was prepared from isolated glomeruli with TRI-Reagent, and 20 μg of total RNA was subjected to Northern blot analysis. (A) Representative Northern blot of TGF-β1 and GAPDH mRNA in indicated experimental periods. (B) Relative ratio of densitometric readings between expressed TGF-β1 and GAPDH mRNA as shown in A. Broken lines represent relative ratio of 1 for the NC group. Values are given as mean ± SEM, and *n* is the number of animals examined. Abbreviations as in Figure 1. *$P < 0.05$ DD *versus* NC; **$P < 0.05$ DL *versus* DD.

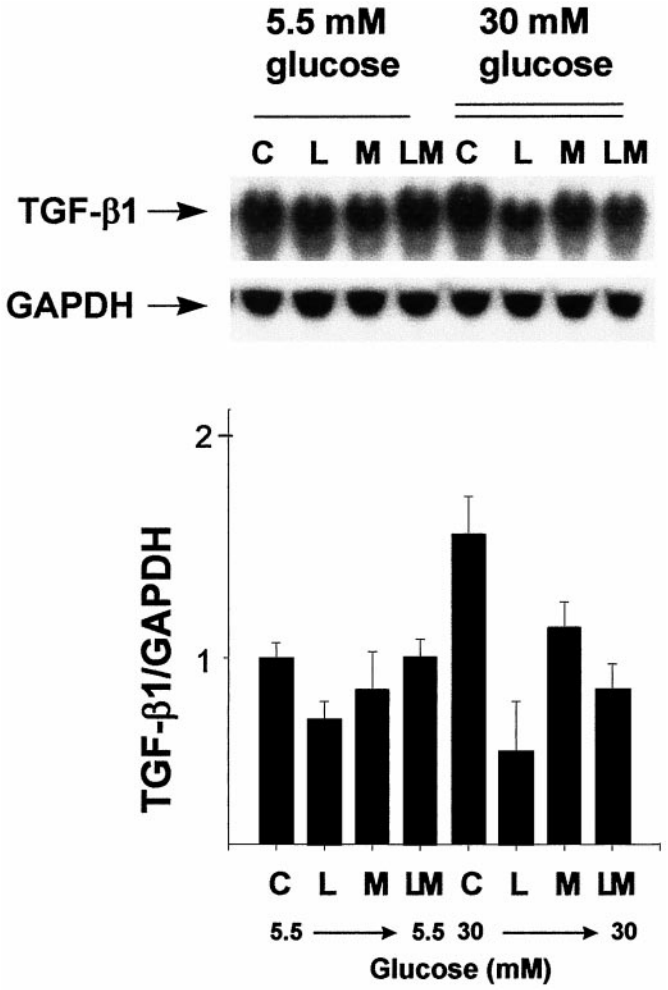

Figure 4. Effect of lovastatin on TGF-β1 mRNA expression in cultured rat mesangial cells. Quiescent rat mesangial cells were treated with serum-free Dulbecco's modified Eagle's medium (DMEM) containing control (5.5 mM) or high (30 mM) glucose for 24 h. At the beginning of treatment with glucose-containing media, lovastatin and mevalonate were added to the culture alone or together (C, control; L, addition of 10 μM lovastatin; M, addition of 100 μM mevalonate; LM, addition of both 10 μM lovastatin and 100 μM mevalonate). After 24-h incubation at 37°C, total RNA was isolated and subjected to Northern blot analysis for TGF-β1 and GAPDH mRNA. Values are expressed as mean ± SEM of four experiments.

suppressed TGF-β1 mRNA expression in mesangial cells cultured under both control and high glucose concentrations. The suppressive effect of lovastatin on TGF-β1 mRNA expression was partially reversed by mevalonate. Western blot for TGF-β in the culture supernatant revealed a unique signal at an apparent molecular weight of about 55 kD, but not at 12.5 kD, the TGF-β monomer under the reducing condition (Figure 5). The amount of this protein changed in parallel with the TGF-β1 mRNA level in response to lovastatin and mevalonate. Although the 12.5-kD TGF-β monomer was not detected in our Western blot presumably because of low concentration, TGF-β activity as measured by mink lung epithelial cell growth inhibition assay was detected in culture media in the absence of treatment to activate the TGF-β produced. The activity in media containing high glucose was slightly higher than that in media containing control glucose (data not shown). However, lovastatin was highly toxic to mink lung epithelial cells and, therefore, TGF-β activity could not be assayed in culture supernatant containing lovastatin. The toxic effect on mink lung epithelial cells was not abolished completely even after dialysis of media with phosphate-buffered saline for 4 d (data not shown).

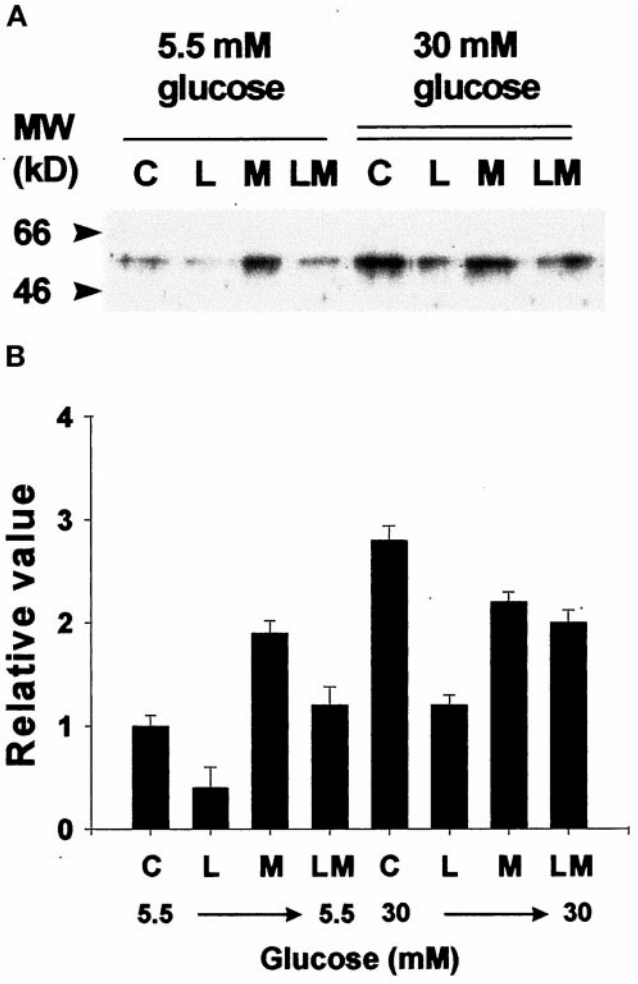

Figure 5. Effect of lovastatin on TGF-β synthesis in cultured rat mesangial cells. Treatment of rat mesangial cells was the same with the method described in Figure 4, but the incubation time was 48 h after treatment of glucose-containing media. One hundred micrograms of proteins in the respective supernatant was applied on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and TGF-β was analyzed by enhanced chemiluminescence (ECL)-linked Western blot with polyclonal TGF-β antibody. (A) Western blots for TGF-β in the respective supernatant; arrowheads indicate molecular size marker proteins (66 kD, bovine serum albumin; 46 kD, ovalbumin). (B) Relative amount of signals presumed to be pro-TGF-β protein. Values are given as mean ± SEM of four experiments.

Effect of Lovastatin on Fibronectin mRNA and Protein Expression in Cultured Rat Mesangial Cells

The expression of fibronectin increased at the protein level as well as the mRNA level under high glucose concentration, as shown in Figures 6 and 7. This increase was markedly reduced by lovastatin. The suppressive effect of lovastatin on fibronectin expression was almost completely reversed by mevalonate. The effects of lovastatin and mevalonate were observed in cells cultured in control glucose as well. The changes in fibronectin expression in response to lovastatin and mevalonate were comparable to those of TGF-β1.

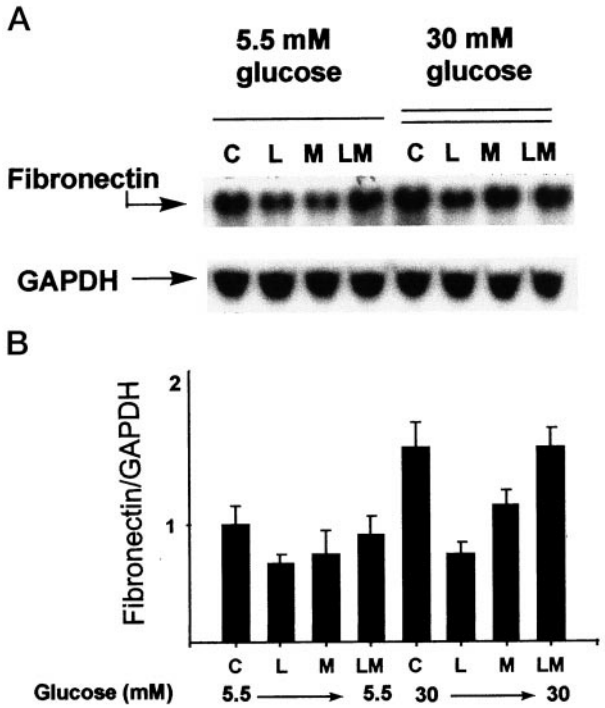

Figure 6. Effect of lovastatin on fibronectin mRNA expression in cultured rat mesangial cells. Quiescent rat mesangial cells were treated with serum-free DMEM containing control (5.5 mM) or high (30 mM) glucose for 24 h. At the beginning of treatment with glucose-containing media, lovastatin and mevalonate were added to the culture alone or together (C, control; L, addition of 10 μM lovastatin; M, addition of 100 μM mevalonate; LM, addition of both 10 μM lovastatin and 100 μM mevalonate). After a 24-h incubation at 37°C, total RNA was isolated and subjected to Northern blot analysis for fibronectin and GAPDH mRNA. Values are expressed as mean ± SEM of four experiments.

Discussion

In this study, glomeruli isolated from streptozotocin-induced diabetic rats and rat mesangial cells cultured under high glucose concentration were used as *in vivo* and *in vitro* models, respectively, to determine the effect of lovastatin, a competitive inhibitor of HMG CoA reductase, on the development and progression of diabetic nephropathy. Untreated diabetic rats exhibited significantly increased UAE, kidney weight, and mean glomerular volume with slight but significant increases in blood cholesterol levels (Table 1). Lovastatin effectively prevented the increase in UAE and renal and glomerular hypertrophy. In untreated diabetic rats, the glomerular TGF-β1 mRNA expression increased up to 1.9-fold of that of control rats, and this increase was completely suppressed by lovastatin. This result suggests that lovastatin may prevent diabetic nephropathy by suppression of glomerular TGF-β1 expression. To confirm the lovastatin effect on the glomerular TGF-β1 expression in diabetic rats, cultured rat glomerular mesangial cells were also examined. Although it was reported previously that TGF-β1 expression was not affected by lovastatin in human mesangial cells (26), we clearly demonstrated that lovastatin suppressed TGF-β1 expression in rat mesangial cells

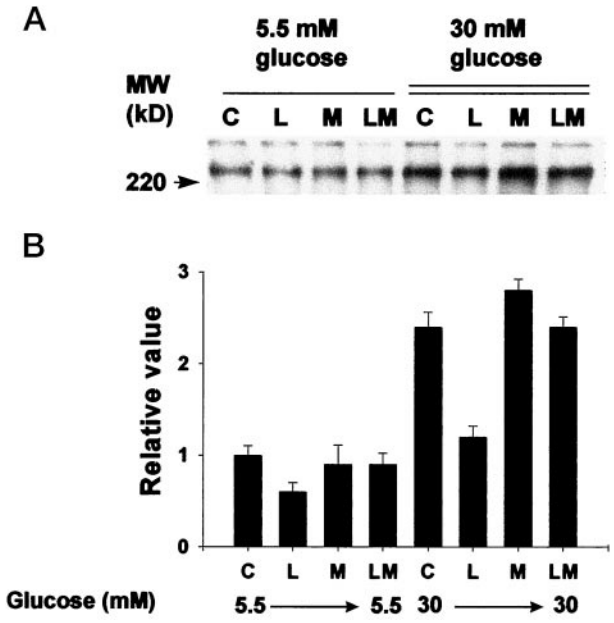

Figure 7. Effect of lovastatin on fibronectin synthesis in cultured rat mesangial cells. Treatment of rat mesangial cells was the same with the method described in Figure 6, but the incubation time was 48 h after treatment of glucose-containing media. Twenty micrograms of proteins in the respective supernatant was applied on 8% SDS-PAGE, and fibronectin was analyzed by ECL-linked Western blot with polyclonal fibronectin antibody. (A) Western blots for fibronectin in the respective supernatant; arrowheads indicate molecular size marker proteins (220 kD, myosin). (B) Relative amount of positive signals. Values are given as mean ± SEM of four experiments.

cultured under both control and high glucose concentrations as well as in diabetic rat glomeruli. HMG CoA reductase, the target enzyme of lovastatin, converts HMG CoA to mevalonate irreversibly, and this reaction is the rate-limiting step in the mevalonate pathway. The inhibition of HMG CoA reductase results in the lack of not only mevalonate, but also a variety of isoprenoid metabolites such as dolichol, ubiquinone, farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP). This deficiency of the metabolites can be overcome by the addition of mevalonate, a key intermediate in this pathway. In this study, the lovastatin effect was nearly completely reversed by mevalonate. Therefore, it is clear that the mevalonate pathway is involved in modulation of the glomerular and mesangial cell TGF-β1 expression by lovastatin.

In Western blot analysis, a single protein with apparent molecular weight of 55 kD was specifically reactive with polyclonal TGF-β antibody, and the amount of this protein changed in parallel with TGF-β1 mRNA levels in response to lovastatin and mevalonate. The activation of TGF-β1 requires cleavage of pro-TGF-β1 (390 amino acid protein) to produce small latent complexes composed of latency-associated protein and 12.5-kD mature TGF-β1 (112 amino acid protein). It has been suggested that the protease responsible for this cleavage might be KEX/furin-like proteases, which recognize multibasic sites in secreted proteins (27). The pro-TGF-β1 has an apparent molecular weight of 45 to 56 kD depending on glycosylation (28) and is usually produced from various cells in serum-free culture media because of deficiency in proteases. Therefore, it is suggested that the 55-kD protein observed in this study is an unprocessed pro-TGF-β.

The possible cellular targets of lovastatin are membrane-bound small GTP binding proteins including Ras and Rho family proteins since isoprenoids such as FPP and GGPP are also metabolites of the mevalonate pathway. These small GTP binding proteins must be bound to membrane (through the protein prenylation [29] such as farnesylation and geranylgeranylation) to become functional in the signaling pathway. Therefore, the inhibition of the mevalonate pathway affects the lipidation and localization of the small GTP binding proteins (30) by limitation of FPP and GGPP. Ras protein is involved in the signaling pathway for cell growth, differentiation, and development. The major signaling pathway following Ras activation is the mitogen-activated protein (MAP) kinase pathway leading to expression of early responsive genes such as *c-fos* and *c-jun*. The heterodimer of c-Fos and c-Jun proteins, so called activator protein-1 (AP-1), is a transcription factor for many genes encoding proteins involved in cell proliferation. Interestingly, there are three different AP-1 binding sites in the promoter region of human TGF-β1 gene (31), and these AP-1 binding sites are the major responsible elements in tetradecanoylphorbol acetate activation and TGF-β1 autoinduction of TGF-β1 gene expression. A recent study showed that Smad3 and Smad4, intracellular signaling molecules involved in TGF-β signaling, cooperate with c-Fos/c-Jun to mediate TGF-β-induced transcription efficiently (32). Therefore, it is reasonable to assume that the reduction of farnesylated Ras by inhibition of the mevalonate pathway with lovastatin possibly suppresses AP-1 (c-Fos/c-Jun) synthesis and results in the suppression of TGF-β1 expression. It is well known that TGF-β is a key mediator of deposition of ECM proteins, including fibronectin under various conditions such as high glucose (5,33). In this context, it is suggested that the suppressive effect of lovastatin on TGF-β expression through the reduction in Ras activity may result in the suppression of ECM synthesis including fibronectin in diabetic glomeruli. Recently, Essig *et al.* (19) proposed that lovastatin may enhance the degradation of ECM proteins by modulating plasminogen activator/plasmin system. In rat proximal tubular cells, limited geranylgeranylation of Rho proteins rather than farnesylation of Ras after lovastatin treatment led to increases in tissue-type plasminogen activator (tPA) and urokinase (uPA) and decreases in plasminogen activator inhibitor-1 (PAI-1) through reorganization of cytoskeleton (19). This was the first demonstration that lovastatin increases ECM-degrading protease activity.

Our *in vivo* and *in vitro* results suggest that HMG CoA reductase inhibition by lovastatin delays the onset and progression of diabetic glomerular injury in experimental diabetes by suppressing glomerular TGF-β and fibronectin expression. Although the precise mechanism involved in the lovastatin effect on ECM proteins at the cellular and molecular level is not known, it appears from this and other studies that lovastatin decreases lipidation of Ras and/or Rho proteins leading to

suppression of TGF-β1 and PAI-1 expression and an increase in ECM-degrading enzymes such as tPA and uPA which, in turn, reduce accumulation of ECM proteins.

Acknowledgments

This work was supported by a grant from Korea Science and Engineering Foundation (KOSEF 94-0403-07-01-3). The authors thank Choong Wae Pharmaceutical Co. (Seoul, Korea) for the generous gift of lovastatin. The authors acknowledge the technical support of Dr. Mi Kyung Cha, Dr. Jeong Ho Kim, Dr. Kyung Il Song, Mr. Dong Cheol Kim, Mrs. Hoo Nam Choi, Miss Hyun Seung Kang, and Miss Mi Ho Kim.

References

1. Mauer SM: Structural-functional relationships in diabetic nephropathy. *Kidney Int* 74: 1143–1155, 1984
2. Ziyadeh FN, Snipes ER, Watanabe M, Alvarez RJ, Goldfarb S, Haverty TP: High glucose induces cell hypertrophy and stimulates collagen gene transcription in proximal tubule. *Am J Physiol* 259: F704–F714, 1990
3. Ayo SH, Radink RA, Glass WF II, Garoni JA, Rampt ER, Appling DR, Kreisberg JI : Increased extracellular matrix synthesis and mRNA in mesangial cells grown in high-glucose medium. *Am J Physiol* 260: F185–F191, 1991
4. Fukui M, Nakamura T, Egihara I, Shirato I, Tomino Y, Koide H: Extracellular matrix component gene expression and its modulation by insulin in diabetic rats. *Diabetes* 41: 1520–1527, 1992
5. Oh JH, Ha H, Yu MR, Lee HB: Sequential effects of high glucose on mesangial cell TGF-β1 and fibronectin synthesis. *Kidney Int* 54: 1872–1878, 1998
6. Rocco MV, Chen Y, Goldfarb S, Ziyadeh FN: Elevated glucose stimulates TGF-β gene expression and bioactivity in proximal tubule. *Kidney Int* 41: 107–114, 1992
7. Kopp JB, Factor VM, Mozes M, Nagy P, Sanderson N, Bottinger EP, Klotman PE, Thorgeirsson SS: Transgenic mice with increased plasma levels of TGF-β1 develop progressive renal disease. *Lab Invest* 74: 991–1003, 1996
8. Sharma K, Ziyadeh FN: The emerging role of transforming growth factor-β in kidney diseases. *Am J Physiol* 35: F829–F842, 1994
9. Laiho M, Saksela O, Andreasen PA, Keski-Oja J: Enhanced production and extracellular deposition of the endothelial-type plasminogen activator inhibitor in cultured human lung fibroblasts by transforming growth factor-β. *J Cell Biol* 103: 2403–2410, 1986
10. Border WA, Noble NA: Evidence that TGF-β should be a therapeutic target in diabetic nephropathy [Editorial]. *Kidney Int* 54: 1390–1391, 1998
11. Kasiske BL, O'Donnell MP, Garvis WJ, Keane WF: Pharmacologic treatment of hyperlipidemia reduces glomerular injury in the rat 5/6 nephrectomy model of chronic renal failure. *Circ Res* 62: 367–374, 1988
12. Lee SK, Jin SY, Han DC, Hwang SD, Lee HB: Effects of delayed treatment with enalapril and/or lovastatin on the progression of glomerulosclerosis in 5/6 nephrectomized rats. *Nephrol Dial Transplant* 8: 1338–1343, 1993
13. Kasiske BL, O'Donnell MP, Cleary MP, Keane WF: Treatment of hyperlipidemia reduces glomerular injury in obese Zucker rats. *Kidney Int* 33: 667–672, 1988
14. Han DC, Lee SK, Hwang SD, Lee HB: Lovastatin prevents glomerulosclerosis in streptozotocin-induced diabetic uninephrectomized rats [Abstract]. *Kidney Int* 44: 1181, 1993
15. O'Donnell MP, Kasiske BL, Kim Y, Atluru D, Keane WF: Lovastatin inhibits proliferation of rat mesangial cells. *J Clin Invest* 91: 83–87, 1993
16. Ghosh PM, Mott GE, Ghosh-Choudhury N, Radnik RA, Stapleton ML, Ghidoni JJ, Kreisgerg JI: Lovastatin induces apoptosis by inhibiting mitotic and post-mitotic events in cultured mesangial cells. *Biochim Biophys Acta* 1359: 13–24, 1997
17. Guijarro C, Kim Y, Schoonover CM, Massy ZA, O'Donnell MP, Kasiske BL, Keane WF, Kashtan CE: Lovastatin inhibits lipopolysaccharide-induced NF-kappa B activation in human mesangial cells. *Nephrol Dial Transplant* 11: 990–996, 1996
18. Guijarro C, Keane WF: Effects of lipids on the pathogenesis of progressive renal failure: Role of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors in the prevention of glomerulosclerosis. *Miner Electrolyte Metab* 22: 147–152, 1996
19. Essig M, Vrtovsnik F, Nguyen G, Sraer JD, Friedlander G: Lovastatin modulates in vivo and in vitro the plasminogen activator/plasmin system of rat proximal tubular cells: Role of geranylgeranylation and Rho proteins. *J Am Soc Nephrol* 9: 1377–1388, 1998
20. Han DC, Kim JH, Cha MK, Song KI, Hwang SD, Lee HB: Effect of HMG Co A reductase inhibition on TGF-β1 mRNA expression in diabetic rat glomeruli. *Kidney Int* 48[Suppl 51]: S61–S65, 1995
21. Weibel ER: Practical methods for biological morphometry. In: *Stereological Methods*, London, Academic Press, 1979, pp 44–45
22. Lee HB, Cha MK, Song KI, Kim JH, Lee EY, Kim SI, Kim J, Yoo MH: Pathogenic role of advanced glycosylation end products in diabetic nephropathy. *Kidney Int* 52[Suppl 60]: S60–S65, 1997
23. Lane PH, Steffes MW, Mauer SM: Estimation of glomerular volume: A comparison of four methods. *Kidney Int* 41: 1085–1089, 1992
24. Harper PA, Robinson JM, Hoover RL, Wright TC, Karnovsky MJ: Improved methods for culturing rat glomerular cells. *Kidney Int* 26: 875–880, 1984
25. Jakobisiak M, Bruno S, Skierski JS, Darzynkiewicz Z: Cell cycle-specific effects of lovastatin. *Proc Natl Acad Sci USA* 88: 3628–3632, 1991
26. Guijarro C, O'Donnell MP, Kasiske BL, Kim Y, Atluru D, Keane WF: Differential effects of lovastatin on human mesangial cell mRNA for cytokines involved in proliferation and matrix turnover [Abstract]. *J Am Soc Nephrol* 4: 770, 1993
27. Barr E, Leiden JM: Systemic delivery of recombinant proteins by genetically modified myoblasts. *Science* 254: 1507–1509, 1991
28. Madisen L, Lioubin MN, Farrand AL, Brunner AM, Purchio AF: Analysis of proteolytic cleavage of recombinant TGF-β1: Production of hybrid molecules with increased processing efficiency. *Ann NY Acad Sci* 593: 7–25, 1990
29. Casey PJ: Protein lipidation in cell signaling. *Science* 268: 221–225, 1995
30. Kim SI, Lee HB: Effect of lovastatin on mesangial cell synthesis of TGF-β1 and fibronectin [Abstract]. *J Am Soc Nephrol* 9: 635A, 1998
31. Kim S-J, Angel P, Lafyatis R, Hattori K, Kim KY, Sporn MB, Karin M, Robers AB: Autoinduction of transforming growth factor β1 is mediated by the AP-1 complex. *Mol Cell Biol* 10: 1492–1497, 1990
32. Zhang Y, Feng XH, Deryneck R: Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TGF-beta-induced transcription. *Nature* 394: 909–913, 1998
33. 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