

# IGF-1 Decreases Collagen Degradation in Diabetic NOD Mesangial Cells

## Implications for Diabetic Nephropathy

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**Nonobese diabetic (NOD) mice develop glomerulosclerosis shortly after the onset of diabetes. We showed that mesangial cells (MCs) from diabetic mice exhibited a stable phenotypic switch, consisting of both increased IGF-1 synthesis and proliferation (Elliot SJ, Striker LJ, Hattori M, Yang CW, He CJ, Peten EP, Striker GE: Mesangial cells from diabetic NOD mice constitutively secrete increased amounts of insulin-like growth factor-I. *Endocrinology* 133:1783–1788, 1993). Because the extracellular matrix (ECM) accumulation in diabetic glomerulosclerosis may be partly due to decreased degradation, we examined the effect of excess IGF-1 on collagen turnover and the activity of metalloproteinases (MMPs) and tissue inhibitors of metalloproteinase (TIMPs) in diabetic and nondiabetic NOD-MC. Total collagen degradation was reduced by  $58 \pm 18\%$  in diabetic NOD-MCs, which correlated with a constitutive decrease in MMP-2 activity and mRNA levels, and nearly undetectable MMP-9 activity and mRNA. TIMP levels were slightly decreased in diabetic NOD-MC. The addition of recombinant IGF-1 to nondiabetic NOD-MC resulted in a decrease in MMP-2 and TIMP activity. Furthermore, treatment of diabetic NOD-MC with a neutralizing antibody against IGF-1 increased the latent form, and restored the active form, of MMP-2. In conclusion, the excessive production of IGF-1 contributes to the altered ECM turnover in diabetic NOD-MC, largely through a reduction of MMP-2 activity. These data suggest that IGF-1 could be a major contributor to the development of diabetic glomerulosclerosis. *Diabetes* 48:1638–1644, 1999**

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ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GH, growth hormone; MC, mesangial cell; MMP, metalloproteinase; NIH, National Institutes of Health; PCR, polymerase chain reaction; RT, reverse transcription; STZ, streptozotocin; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1; TIMP, tissue inhibitor of metalloproteinase.

**D** iabetic glomerulosclerosis, defined as an accumulation of extracellular matrix (ECM), is the most common cause of end-stage renal disease (1–4). The local events leading to the glomerular lesions are still incompletely elucidated. We have focused on the nonobese diabetic (NOD) mouse model, since these mice spontaneously develop immune-mediated type 1 diabetes and diabetic nephropathy (5,6). The early stages of the nephropathy, detectable shortly (8 weeks) after the onset of diabetes, consist of glomerular hypertrophy with modest glomerulosclerosis and albuminuria (6). We found that the glomeruli of diabetic NOD mice contained increased laminin, tenascin, and transforming growth factor (TGF)- $\beta$ 1 mRNAs, and an accumulation of tenascin and laminin (7).

IGF-1 is among the candidate molecules that may play a role in the progression of the renal lesions, since the development of renal hypertrophy in diabetic NOD mice has been shown to be associated with the persistent accumulation of IGF-1 (8). We studied mesangial cell lines isolated from NOD mice (NOD-MC), before and after the onset of type 1 diabetes (9). NOD-MC derived from diabetic mice showed a stable phenotypic change consisting of increased IGF-1 secretion and enhanced growth rate (9). This phenomenon has also been reported in other cell types (10–14). We hypothesized that these stable phenotypic alterations may also be present in vivo and could favor the progression of diabetic nephropathy.

Because ECM turnover is altered in diabetic glomerulosclerosis (15,16), ECM synthesis and degradation were compared in diabetic and nondiabetic NOD-MC. Diabetic NOD-MC showed a constitutive decrease in metalloproteinase (MMP)-2 and an almost complete disappearance of MMP-9 activity. These changes were at least partially attributable to decreased transcription, since MMP-2 mRNA levels were decreased and MMP-9 mRNA was undetectable. Tissue inhibitor of metalloproteinase (TIMP) levels, assessed by reverse zymography, were also decreased, but to a lesser extent in diabetic cells. The significance of these findings was confirmed by the finding that conditioned medium from diabetic NOD-MC contained much lower collagenolytic activity than medium from nondiabetic NOD-MC. The role of IGF-1 in ECM degradation was examined in diabetic NOD-MC by neutralizing the excess IGF-1 with neutralizing antibody. Addition of the antibody to diabetic NOD-MC resulted in elevated MMP-2 activity (pro-MMP form) and the restoration of its active form. Furthermore, treatment of nondiabetic NOD-MC

with recombinant IGF-1 induced a decrease in MMP-2 and TIMP activity. These data suggest that IGF-1 may play a determinant role in the development of diabetic glomerulosclerosis in NOD mice by decreasing ECM degradation.

## RESEARCH DESIGN AND METHODS

**Reagents.** Tissue culture plates were obtained from Costar (Cambridge, MA). Collaborative Biomedical (Bedford, MA) provided fibronectin. Fetal bovine serum (FBS) was supplied by Gibco BRL (Grand Island, NY). Recombinant human IGF-1 and a specific neutralizing monoclonal antibody anti-human IGF-1 were obtained from Upstate Biotechnology (Lake Placid, NY). An irrelevant, isotype-matched antibody (anti-rabbit IgG) was purchased from Sigma Chemical (St. Louis, MO). Novex (San Diego, CA) supplied 10% gelatin polyacrylamide zymography gels. Recombinant gelatinase A was a gift from Dr. W. Stetler-Stevenson (National Cancer Institute, Bethesda, MD). Purified human type IV N-[propionate-2,3-<sup>3</sup>H]-propionylated collagen was purchased from Dupont NEN (Boston, MA). Rabbit anti-mouse type I and type IV collagens were from Biodesign (Kennebunk, ME). The TRI reagent was obtained from Molecular Research Center (Cincinnati, OH). The First Strand cDNA synthesis kit was from Boehringer Mannheim (Indianapolis, IN). **Cell culture.** Mesangial cell lines were isolated from diabetic and nondiabetic NOD mice, as previously reported (9). Briefly, they were grown from glomeruli isolated from kidneys of 2- to 4-month-old female diabetic and nondiabetic mice. Diabetic NOD mice had glycosuria for 4–8 weeks before death, and were receiving 2 insulin injections/day (0.5 U of an insulin/NPH mixture). The dose of insulin was adjusted according to urine glycosuria. Nondiabetic NOD glomeruli were obtained from NOD mice that had a normal glucose tolerance test at the time of death. Microdissected glomeruli were plated in separate fibronectin-coated wells of 24-well tissue culture plates. Mesangial-like cellular outgrowths were patch-cloned, propagated, and maintained in basal medium (17) supplemented with 20% FBS, 1 mmol/l glutamine, 0.075% Na<sub>2</sub>HCO<sub>3</sub>, and 6 mmol/l glucose. Mesangial cells (MCs) were identified by morphology and their immunofluorescence staining pattern, as previously described (17). Two lines of diabetic and nondiabetic NOD-MC, derived from different animals, were studied between passages 8–18.

**Experimental conditions.** Because of their different growth rates (9), the number of cells initially plated was adjusted so that the cell number in individual wells was approximately the same at the end of an experiment. Diabetic and nondiabetic NOD-MCs were plated in fibronectin-coated six-well plates at a density of 25,000 and 50,000 cells per well, respectively. After 4–5 days, the cell layers were rinsed three times with serum-free medium and placed in serum-free medium. After 24 h, supernatants were collected and stored at –80°C for the assessment of MMP and TIMP activity.

After trypsinization, cell number was determined by direct cell counting. Total RNA was prepared from a portion of these cells. To evaluate collagen production, cells were grown in fibronectin-coated six-well plates for 4–5 days and then exposed to medium containing 0.1% bovine serum albumin (BSA), 50 µg/ml ascorbic acid, and 80 µg/ml β-aminopropionitrile for 24 h.

In experiments testing the effect of IGF-1–neutralizing antibody, MCs were plated in fibronectin-coated 24-well plates at a density of 10,000 cells per well. The following day, the cells were rinsed three times with serum-free medium and then exposed to medium containing 0.1% FBS. Either increasing concentrations of IGF-1 (1–100 ng/ml), neutralizing antibody (34 µg/ml), or anti-rabbit IgG antibody, as irrelevant antibody (35 µg/ml), were then added to the wells every day for 3 days and the supernatants were collected.

To evaluate the effect of hyperglycemic culture conditions, NOD-MCs were cultured in medium containing 25 mmol/l glucose for 3 weeks, and MMP activity was assessed as described above.

**Zymographic analysis of MMP activity.** The activity of MMPs in conditioned medium from diabetic NOD and nondiabetic NOD-MC was assessed, as previously described (12). Briefly, media were centrifuged to remove cellular debris and diluted appropriately to normalize for cell number. Conditioned media were then mixed with sample buffer (1% SDS, 0.08 M Tris pH 6.8, 4% glycerol, and 0.006% bromophenol blue), and loaded onto a 10% gelatin polyacrylamide gel. After electrophoresis, the gels were incubated in 2.5% Triton X-100 solution for 1 h at room temperature, and then in a collagenase buffer (50 mmol/l Tris pH 7.5, 150 mmol/l NaCl, 10 mmol/l CaCl<sub>2</sub>, and 0.2% Brij-35) at 37°C overnight. Areas of gelatinolytic activity were visualized by staining the gels in a solution containing 0.5% Coomassie brilliant blue, 30% methanol, and 10% acetic acid. The gels were then air-dried, and relative MMP activity was quantitated by densitometry using National Institutes of Health (NIH) Image 1.6. Experimental conditions were assessed before final analysis by assaying different dilutions of conditioned media. Gel strips from selected experiments were also incubated in 50 mmol/l Tris buffer with the addition of 25 mmol/l EDTA to check for non-MMP-dependent bands of gelatinolytic activity.

**Reverse-zymographic analysis of TIMP activity.** The activity of TIMPs in conditioned medium from diabetic NOD and nondiabetic NOD-MC was assessed, as

previously described (18). Briefly, conditioned medium was prepared as for zymography and loaded onto a 15% gelatin polyacrylamide gel containing 0.5% porcine gelatin and 6 µg recombinant gelatinase A. After electrophoresis, the gels were incubated in 2.5% Triton X-100 solution for 3 h at room temperature and then in collagenase buffer at 37°C for 24 h. After staining with Coomassie brilliant blue solution, areas of inhibition of gelatinase A activity were visualized as blue staining regions on a clear background. The gels were then air-dried and relative TIMP activity was quantitated by densitometry using NIH Image 1.6.

**Evaluation of type IV collagen degradation.** Purified human type IV N-[propionate-2,3-<sup>3</sup>H]-propionylated collagen, dissolved in 60% ethanol, was placed in a 96-well tissue culture plate at a concentration of 5 µg/cm<sup>2</sup> (19). After 24 h at room temperature, 50 µl of conditioned medium from diabetic NOD and nondiabetic NOD-MC was added to the wells and the release of [<sup>3</sup>H] into the medium was measured after 16 h of incubation at 37°C.

**Evaluation of type I and IV collagen production by enzyme-linked immuno-sorbent assay.** After 24 h of incubation in serum-free medium containing 0.1% BSA, 50 µg/ml ascorbic acid, and 80 µg/ml β-aminopropionitrile, the supernatants were collected and centrifuged, and protease inhibitors (2.5 mmol/l EDTA, 1 mmol/l phenylmethylsulfonyl fluoride, 10 mmol/l N-ethylmaleimide) were added. To measure cell-associated collagen, cell layers were lysed in 1 ml of 5 mol/l guanidine-HCl/0.1 mol/l Tris (pH 8.6) and protease inhibitors were added. Supernatants and cell lysates were stored at –80°C before assay. Enzyme-linked immunosorbent assay (ELISA) was performed, as previously described (12). The standard range used was 4–64 ng/ml for type I collagen and 2–32 ng/ml for type IV collagen, respectively.

**Reverse transcription–polymerase chain reaction.** Total RNA was prepared using TRI reagent according to the manufacturer's directions. To increase the yield of total RNA, muscle glycogen was used as nonspecific carrier. The final RNA pellet was dissolved in 10 µl of diethylcyanophosphonate water and stored at –70°C; 1 µg of total RNA was reverse transcribed using a First Strand Synthesis kit, as previously described (12), and the final volume was adjusted to 200 µl using DEPC water. In a typical polymerase chain reaction (PCR), 2 µl of reverse transcription (RT) material was used. The expression of mRNA specific for MMP-2, MMP-9, type IV collagen, laminin, and β-actin was analyzed by standard PCR, as previously published (12). Results were quantitated by densitometry using NIH Image 1.6 and expressed in arbitrary densitometric units. We chose β-actin as a housekeeping gene, and used its gene product to normalize RT-PCR data.

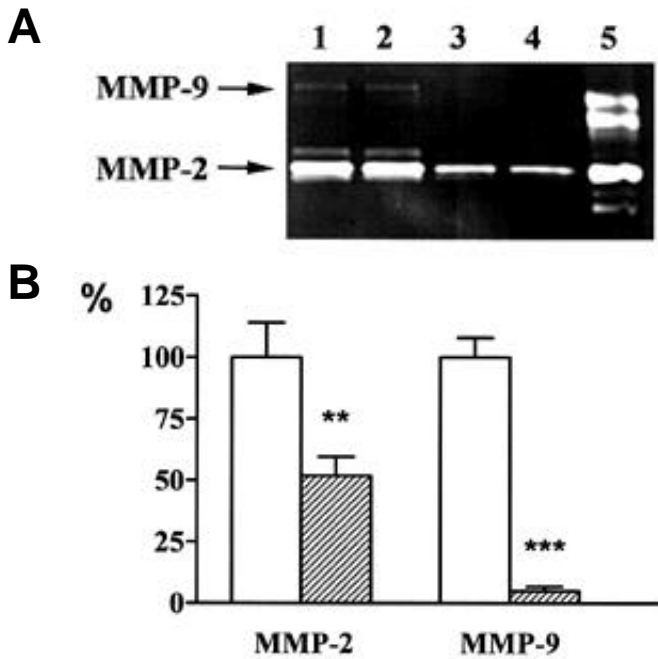
**Data analysis.** Data were collected from three to five independent experiments on duplicate wells. Data are presented as means ± SD. Statistical differences were assessed using Student's *t* test (Prism; GraphPad, San Diego, CA).

## RESULTS

**MMP activity and expression.** Both the latent and active forms of MMP-2 were markedly decreased in supernatants of diabetic NOD-MC (Fig. 1). The active form of MMP-2 was nearly absent in medium from diabetic NOD-MC, whereas the pro-MMP form of the enzyme was decreased by 45.12 ± 26.42% (*P* > 0.01) (Fig. 1B). MMP-9 activity in medium conditioned by diabetic NOD-MC was also virtually undetectable (Fig. 1A). When the zymography gels were incubated in 50 mmol/l Tris buffer containing 25 mmol/l EDTA, no gelatinolytic activity was seen, indicating that the activity present in test gels was due to MMPs (data not shown).

We previously showed that MMP activity was closely related to the mRNA levels in both microdissected glomeruli and MC from bGH transgenic mice (12,20). To determine whether this correlation also existed in NOD-MC, we studied MMP mRNA. The mRNA levels for MMP-2, normalized for β-actin expression, were decreased by 34.90 ± 12.29% in diabetic NOD-MC (Fig. 2), showing that the decrease in MMP-2 activity was at least partially due to decreased gene transcription. Similarly, MMP-9 gene expression was not detectable in diabetic NOD-MC, correlating with the observed lack of MMP-9 activity (Fig. 2).

**TIMP activity.** The activity of the specific endogenous TIMPs was studied by reverse zymography. TIMP-1 and TIMP-2 were detected in conditioned medium from both diabetic and nondiabetic NOD-MC (Fig. 3A). However, both TIMP-1 and TIMP-2 were decreased in diabetic NOD-MC



**FIG. 1. MMP activity.** *A:* Representative zymographic analysis of supernatants from nondiabetic NOD-MC (lanes 1 and 2) and diabetic NOD-MC (lanes 3 and 4). Lane 5: standard. *B:* Comparison of the enzymatic activity for MMP-2 and MMP-9 in the supernatants of nondiabetic NOD-MC (□) and diabetic NOD-MC (▨). Data are expressed as percent of nondiabetic NOD-MC. Shown are means  $\pm$  SD of four independent experiments, carried out in duplicate. \*\* $P < 0.01$ , \*\*\* $P < 0.005$ .

(Fig. 3). In particular, TIMP-1 levels were decreased to  $73.40 \pm 12.84\%$ , and TIMP-2 levels to  $85.64 \pm 3.48\%$ .

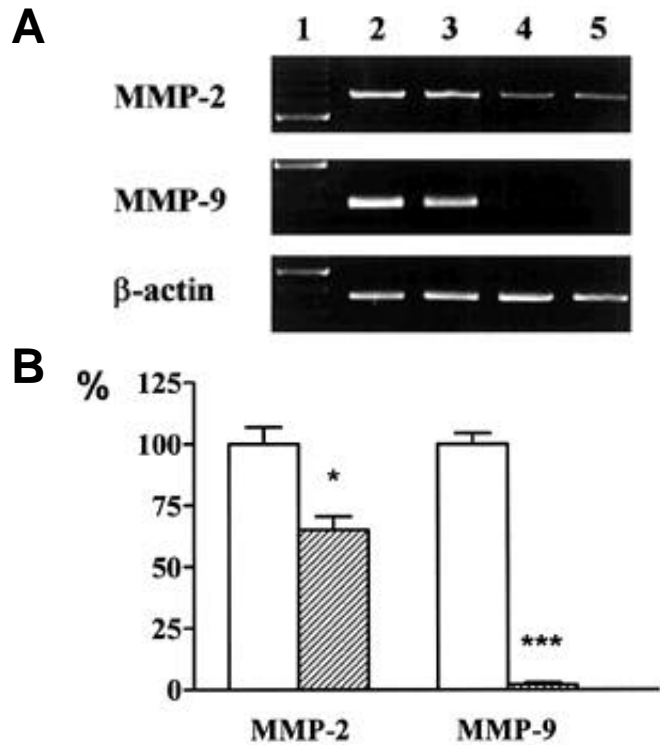
**Collagen degradation.** The net effect of a concomitant reduction in MMP and TIMP activity on collagen degradation in diabetic NOD-MC was examined by measuring total degradative activity in culture supernatants using [ $^3$ H]-labeled type IV collagen. There was a  $58.59 \pm 18.07\%$  decrease in isotope release in media from diabetic cells (Table 1) ( $P < 0.01$ ).

**Collagen production.** There were no differences in type IV ( $153.03 \pm 55.06\%$ ) (Table 1) or type I ( $89.70 \pm 15.63\%$ ) collagen levels in cell lysates and supernatants between diabetic and nondiabetic NOD-MC.

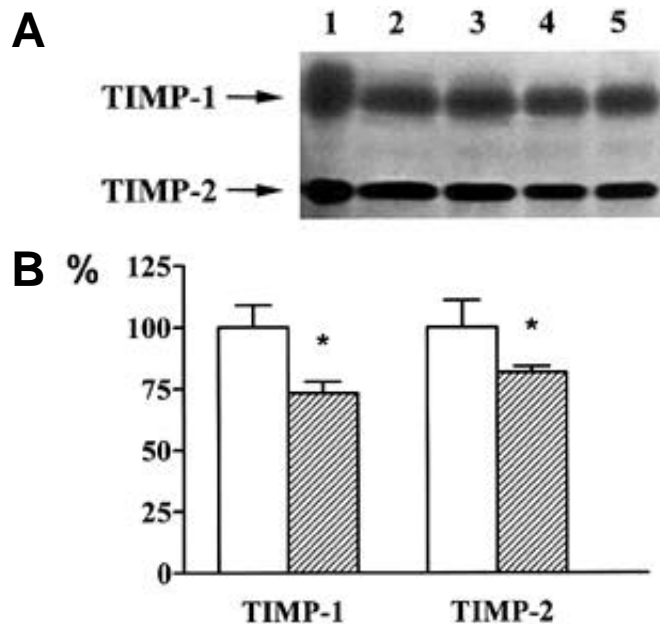
**Type IV collagen and laminin mRNA expression.** Type IV collagen and laminin mRNAs were assessed by semiquantitative PCR and normalized to  $\beta$ -actin expression. The laminin mRNA levels were significantly increased ( $175.21 \pm 46.68\%$ ,  $P < 0.05$ ), whereas the mRNA levels for type IV collagen were unchanged in diabetic NOD-MC cells ( $107.18 \pm 18.34\%$ ) (Table 1).

**Role of IGF-1 in the modulation of MMP and TIMP activity.** Diabetic NOD-MC synthesize and secrete excess amounts of IGF-1 (9). To determine if this excess IGF-1 secretion played a role in the alterations of MMP and TIMP activity found in diabetic NOD-MC, nondiabetic NOD-MC were stimulated with IGF-1. Recombinant human IGF-1 treatment induced a  $26.20 \pm 11.87\%$  ( $P < 0.05$ ) decrease in MMP-2 activity, and a lesser reduction in TIMP-1 ( $15.40 \pm 6.92\%$ ,  $P < 0.05$ ) and TIMP-2 ( $18.73 \pm 7.12\%$ ,  $P < 0.05$ ) levels in nondiabetic NOD-MC (Fig. 4).

Diabetic NOD-MC were treated with neutralizing IGF-1 antibody to provide an additional assessment of the effect of



**FIG. 2. MMP-2 and MMP-9 mRNA expression.** *A:* Representative PCR gels of MMP-2, MMP-9, and  $\beta$ -actin. Lane 1: molecular weight standards; lanes 2 and 3: nondiabetic NOD-MC; lanes 4 and 5: diabetic NOD-MC. *B:* Comparison of the expression of MMP-2 and MMP-9 mRNA in nondiabetic NOD-MC (□) and diabetic NOD-MC (▨). Data are expressed as percent of nondiabetic NOD-MC. Shown are means  $\pm$  SD of three independent experiments carried out in duplicate. \* $P < 0.05$ , \*\*\* $P < 0.005$ .



**FIG. 3. TIMP activity.** *A:* Representative reverse-zymographic analysis of supernatants from nondiabetic NOD-MC (lanes 2 and 3) and diabetic NOD-MC (lanes 4 and 5). Lane 1: standard. *B:* Comparison of TIMP activity in the supernatants of nondiabetic NOD-MC (□) and diabetic NOD-MC (▨). Data are expressed as percent of nondiabetic NOD-MC. Shown are means  $\pm$  SD of three independent experiments carried out in duplicate. \* $P < 0.05$ .

TABLE 1  
Assessment of collagen metabolism in NOD-MC

	Ratio of diabetic to nondiabetic (%)	P value
Type IV collagen degradation	58.59 ± 18.07	<0.01
Type IV collagen production	153.03 ± 55.06	NS
Type IV collagen mRNA expression	107.18 ± 18.34	NS

Data are means ± SD of four independent experiments carried out in duplicate. Comparison of in vitro type IV collagen degradative ability, collagen production, and type IV collagen mRNA expression in nondiabetic and diabetic NOD-MC is shown here. Total degradative activity in the culture supernatants was examined by using <sup>3</sup>H-labeled type IV collagen. Type IV collagen production was studied by ELISA, and type IV collagen mRNA expression was evaluated by RT-PCR. Results are expressed as percent of controls (nondiabetic NOD-MC).

IGF-1 on ECM degradation. This treatment resulted in a 22.23 ± 10.25% increase in the activity of the latent form of MMP-2 and the restoration of the active form of this enzyme (Fig. 5A, B, and C). No change in MMP-9 activity was observed (data not shown). The lack of an effect by IGF-1-neutralizing antibody provided additional evidence that the low MMP-9 levels in diabetic NOD-MC were not directly related to IGF-1 levels in the medium. There was also an increase in TIMP-1 and -2 levels in diabetic NOD-MC (Fig. 6A, B, and C), consistent with an important role of IGF-1 in regulating both MMP-2 and TIMP activities in mesangial NOD-MC. No effect was observed using an irrelevant, isotype-matched antibody (anti-rabbit IgG).

**Effect of hyperglycemic culture conditions on MMP activity.** Nondiabetic and diabetic NOD-MC grown in 25 mmol/l glucose-containing medium for 3 weeks showed a significant decrease in MMP-2 activity (16.7 ± 5.8% for nondiabetic and 19.5 ± 6.7% for diabetic NOD-MC, respectively,  $P < 0.05$ ; data not shown). No effect on MMP-9 activity was observed.

## DISCUSSION

MMPs are thought to be important participants in the turnover of ECM in the kidney and other tissues in both normal and disease states (16,20–31). Several lines of evidence suggest that decreased degradation of ECM components plays a role in diabetic nephropathy. MMP activity is reduced in glomeruli of rats with streptozotocin (STZ)-induced diabetes and in some other animal models of renal fibrosis (16,29–31). The expression of MMP-2 and MMP-9 has been reported in quiescent mouse (20), rat (32), and human (33) mesangial cell lines. Exposure of MC to high glucose concentrations downregulates the expression of MMP-2 in rat (34) and human MC (35). This is consistent with the results of the present study, which showed a decrease in both the gene expression and the biologic activity of MMP-2 (–46% in pro-MMP form and absence of the active form) and MMP-9 (absent) in diabetic cells. This altered phenotype was present in cell lines derived from several different mice, and at all culture passages examined, suggesting that the cells acquired the modified phenotype in vivo. The cells from diabetic NOD mice also showed a constitutive decrease in the activity of TIMP-1 and -2, which are specific endogenous inhibitors of MMPs. Since this phenom-

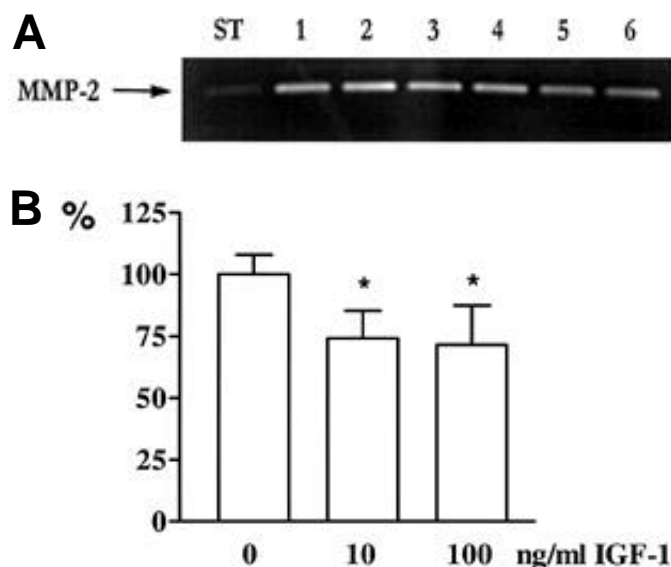
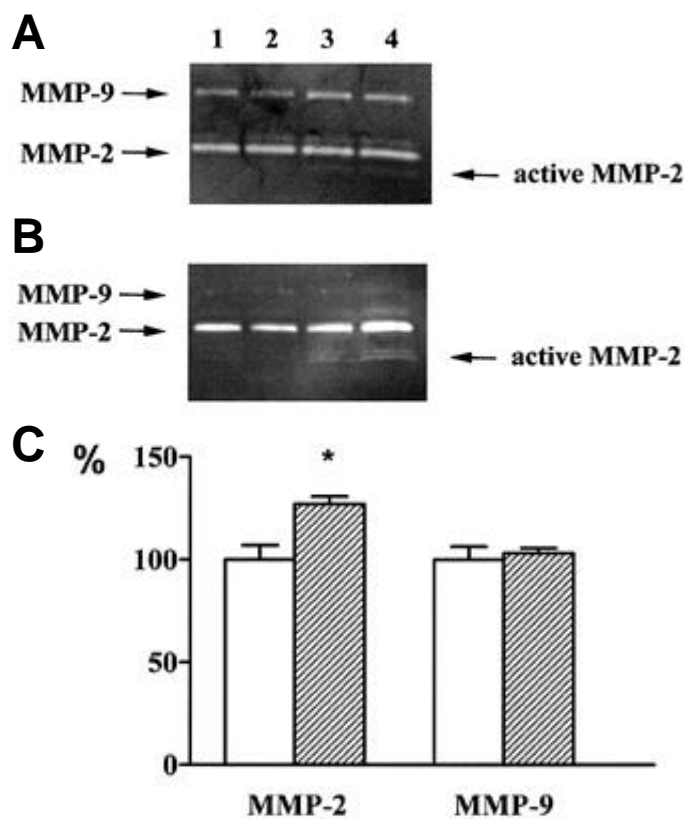


FIG. 4. Effect of IGF-1 on MMP-2 activity. A: Representative zymographic analysis of MMP-2 activity in supernatants from nondiabetic NOD-MC treated with increasing concentrations of IGF-1. Lanes 1 and 2: unstimulated cells; lanes 3 and 4: 10 ng/ml IGF-1; lanes 5 and 6: 100 ng/ml IGF-1. B: Comparison of the enzymatic activity for MMP-2 in the supernatants of nondiabetic NOD-MC treated with increasing concentrations of IGF-1. Data are expressed as percent of unstimulated nondiabetic NOD-MC. Shown are means ± SD of three independent experiments carried out in duplicate. \* $P < 0.05$ . ST, standard.

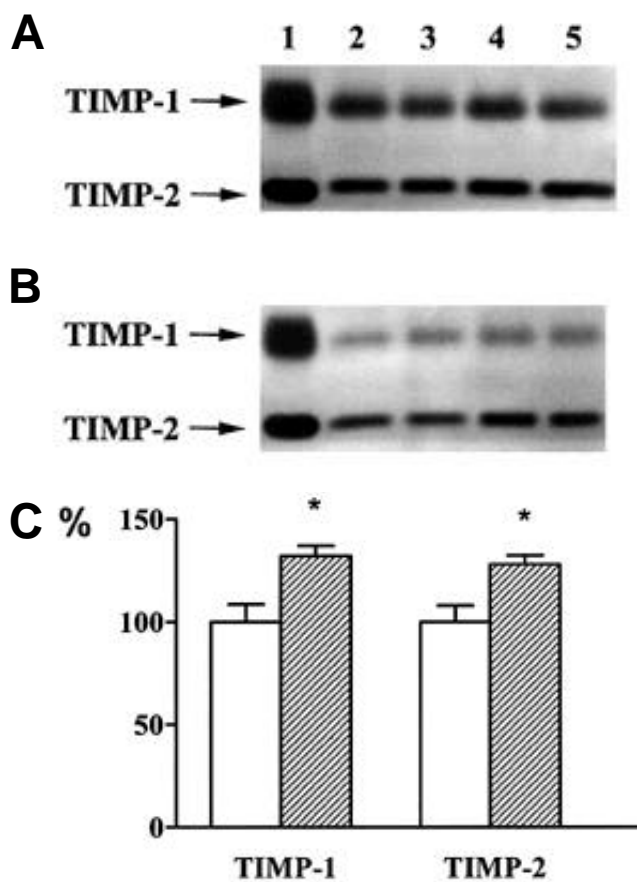
enon could counterbalance the decrease in MMP activity, we evaluated the ability of conditioned medium to degrade <sup>3</sup>H-type IV collagen in vitro. We found that collagen degradation was markedly reduced in diabetic, compared with nondiabetic, NOD-MC. However, the net accumulation of type I and IV collagens did not differ significantly between diabetic and nondiabetic NOD-MC over a 72-h period of incubation. In addition, the mRNA levels were not different between prediabetic and postdiabetic NOD-MC. These in vitro data suggest that the accumulation of type IV collagen in the mesangial regions of the glomeruli of diabetic NOD mice may be a consequence of decreased degradation (6). This interpretation is consistent with our previous findings that tenascin, laminin, and type IV collagen accumulated in the mesangial regions of the glomeruli of diabetic NOD mice (6,7). Because MMPs degrade a relatively broad spectrum of substrates, reduced MMP activity may be an important determinant of diabetic nephropathy, favoring the glomerular accumulation of ECM components. Although the changes found in response to alterations in IGF-1 levels were modest at the time points studied, one might not expect large changes in ECM turnover, since diabetic glomerulosclerosis develops over a period of decades. This study focused on MMP-2 and MMP-9, because they are the principal enzymes involved in collagen degradation in the mesangium. Other MMPs are also present in the mesangium, but their principal substrates are noncollagenous components of the ECM (25–33).

We had previously shown that MC isolated from diabetic NOD mice had increased IGF-1 synthesis (9). This led us to examine whether IGF-1 contributed to the diabetic phenotype. In the present study, we conclude that the constitutive decrease in MMP-2 activity in the diabetic cells may be attrib-



**FIG. 5.** Effect of IGF-1-neutralizing antibody on MMP activity. **A:** Representative zymographic analysis of supernatants from nondiabetic NOD-MC treated with IGF-1-neutralizing monoclonal antibody (34  $\mu$ g/ml) (lanes 3 and 4) and an irrelevant isotype-matched monoclonal antibody (anti-rabbit IgG, 34  $\mu$ g/ml) (lanes 1 and 2). **B:** Representative zymographic analysis of supernatants from diabetic NOD-MC treated with IGF-1-neutralizing monoclonal antibody (34  $\mu$ g/ml) (lanes 3 and 4) and an irrelevant isotype-matched monoclonal antibody (anti-rabbit IgG, 34  $\mu$ g/ml) (lanes 1 and 2). **C:** Comparison of the enzymatic activity for MMP-2 ( $\square$ ) and MMP-9 ( $\boxtimes$ ) in the supernatants of diabetic NOD-MC treated with IGF-1-neutralizing monoclonal antibody (34  $\mu$ g/ml) and an irrelevant isotype-matched monoclonal antibody (anti-rabbit IgG, 34  $\mu$ g/ml), expressed as percent of diabetic NOD-MC treated with anti-rabbit IgG. Shown are means  $\pm$  SD of three independent experiments carried out in duplicate. \* $P < 0.05$ .

uted to the increased secretion of IGF-1. This conclusion is based on two findings. First, the addition of recombinant IGF-1 to nondiabetic NOD-MC induced a decrease ( $-26\%$ ) in MMP-2 activity. Second, treatment of diabetic NOD-MC with IGF-1-neutralizing antibody induced an increase (22%) of the MMP-2 activity (pro-MMP form) and the restoration of the active form of this enzyme. Thus, the MMP-2 response to the addition of IGF-1 to nondiabetic NOD-MC or the addition of a neutralizing antibody to diabetic NOD-MC provides strong evidence that IGF-1 plays a determinative role in the changes found in NOD-MC after the onset of diabetes in vivo. The addition of an IGF-1-neutralizing antibody had no effect on MMP-9 activity in diabetic NOD-MC, effectively excluding a role for IGF-1 in the disappearance of MMP-9 in these cells. This suggests that the regulation of MMP-2 and MMP-9 are not linked, a conclusion in accordance with the observations that they are upregulated by interleukin- $1\beta$ , tumor necrosis factor- $\alpha$ , or phorbol myristate acetate through different signaling pathways (36,37). In confirmation of this conclusion is the obser-



**FIG. 6.** Effect of IGF-1-neutralizing antibody on TIMP activity. **A:** Representative reverse-zymographic analysis of supernatants from nondiabetic NOD-MC treated with IGF-1-neutralizing monoclonal antibody (34  $\mu$ g/ml) (lanes 4 and 5) and an irrelevant isotype-matched monoclonal antibody (anti-rabbit IgG, 34  $\mu$ g/ml) (lanes 2 and 3). **Lane 1:** standard. **B:** Representative reverse-zymographic analysis of supernatants from diabetic NOD-MC treated with IGF-1-neutralizing monoclonal antibody (34  $\mu$ g/ml) (lanes 4 and 5) and an irrelevant isotype-matched monoclonal antibody (anti-rabbit IgG, 34  $\mu$ g/ml) (lanes 2 and 3). **Lane 1:** standard. **C:** Comparison of the activity of TIMP-1 ( $\square$ ) and TIMP-2 ( $\boxtimes$ ) in the supernatants of diabetic NOD-MC treated with IGF-1-neutralizing monoclonal antibody (34  $\mu$ g/ml) and an irrelevant isotype-matched monoclonal antibody (anti-rabbit IgG, 34  $\mu$ g/ml). Data are expressed as percent of diabetic NOD-MC treated with anti-rabbit IgG. Shown are means  $\pm$  SD of three independent experiments carried out in duplicate. \* $P < 0.05$ .

vation that MMP-9 expression was induced in the injured remodeling cornea (38), as well as in migrating oral mucosa epithelium and granulation tissue, while MMP-2 levels remained stable (39). A causal relationship between IGF-1 stimulation and reduced collagenase production was previously reported in bone cell and fibroblast cultures (40,41). To our knowledge, this is the first study to demonstrate a direct effect of IGF-1 on MMP and TIMP activity in cultured MC.

Multiple studies suggest a role of the GH/IGF-1 axis in the renal complications of diabetes (for a review, see 42 and 43). Increased renal tissue concentrations of IGF-1 have been described in STZ-induced diabetic rats (43,44) and more recently in diabetic NOD mice (8). Moreover, a strong association of urinary IGF-1 with kidney volume, a marker for glomerular hypertrophy, and of both urinary IGF-1 and GH with microalbuminuria has been shown in patients with type 1

diabetes (45). The mechanisms of IGF-1 accumulation in the kidneys are not completely understood. However, an increase in IGF-1 protein content was not associated with a concomitant increase in mRNA levels for IGF-1 in diabetic NOD mice (8) or in rats with STZ-induced diabetes (46,47). While this discrepancy might be explained by an increase in IGF-1 mRNA transcript stability or posttranslational modifications in IGF-1 protein stability, there is no experimental evidence to support this theory. Other mechanisms potentially leading to the local accumulation or enhanced biological activity of IGF-1 involve changes in the expression of IGF-1 receptor (8,48) or alterations in the renal IGF binding proteins (IGFBP) mRNA distribution and levels (8,43,47,48).

Several observations suggest that cells from glomeruli of mice with glomerulosclerosis may undergo irreversible phenotypic changes that are maintained in vitro. For instance, we previously reported that MCs derived from bGH-transgenic mice lacked MMP-9 (12). Interestingly, both in MCs derived from bGH-transgenic and from diabetic NOD mice, a significant reduction in MMP-9 levels has been found at the mRNA and protein levels. Others have compared MCs derived from rat strains with a different propensity to develop renal lesions (13) or fibroblasts from diabetic and nondiabetic patients (14). In all these different cell types, the change acquired in vivo was stably expressed in vitro. In the current study, we were able to induce partial reversion of the altered phenotype, toward the nondiabetic profile, by treating diabetic NOD-MC with IGF-1-neutralizing antibody. This result stresses the importance of better characterizing the nature of this switch at the cellular and molecular level to design new therapeutic approaches.

## REFERENCES

- Morel-Maroger Striker L, Killen P, Chi E, Striker GE: The composition of glomerulosclerosis: studies in focal sclerosis, crescentic glomerulosclerosis, and membranoproliferative glomerulonephritis. *Lab Invest* 51:181-192, 1984
- Adler S, Striker LJ, Striker GE, Perkinson DT, Hibbert J, Couser WG: Studies on progressive glomerular sclerosis in the rat. *Am J Pathol* 123:553-562, 1986
- Steffes MW, Osterby R, Chavers B, Mauer SM: Mesangial expansion as a central mechanism for loss of kidney function in diabetic patients. *Diabetes* 38:1077-1081, 1989
- Striker LJ, Doi T, Elliot SJ, Striker GE: The contribution of glomerular mesangial cells to progressive glomerulosclerosis. *Semin Nephrol* 9:318-328, 1989
- Tochino Y: The NOD mouse as a model of type I diabetes. *Crit Rev Immunol* 8:49-81, 1987
- Doi T, Hattori M, Agodoa LY, Sato T, Yoshida H, Striker LJ, Striker GE: Glomerular lesions in nonobese diabetic mouse: before and after the onset of hyperglycemia. *Lab Invest* 63:204-212, 1990
- Yang CW, Hattori M, Vlassara H, He CJ, Carome MA, Yamato E, Elliot SJ, Striker GE, Striker LJ: Overexpression of transforming growth factor- $\beta$ 1 is associated with up-regulation of glomerular tenascin and laminin gene expression in nonobese diabetic mice. *J Am Soc Nephrol* 5:1610-1617, 1995
- Segev Y, Landau D, Marbach M, Shehadeh N, Flyvbjerg A, Phillip M: Renal hypertrophy in hyperglycemic non-obese diabetic mice is associated with persistent renal accumulation of insulin-like growth factor-I. *J Am Soc Nephrol* 8:436-444, 1997
- Elliot SJ, Striker LJ, Hattori M, Yang CW, He CJ, Peten EP, Striker GE: Mesangial cells from diabetic NOD mice constitutively secrete increased amounts of insulin-like growth factor-I. *Endocrinology* 133:1783-1788, 1993
- Roy S, Sala R, Cagliero E, Lorenzi M: Overexpression of fibronectin induced by diabetes or high glucose: phenomenon with a memory. *Proc Natl Acad Sci U S A* 87:404-408, 1990
- Mecham RP, Whitehouse LA, Wrenn DS, Parks WC, Griffin GL, Senior RM, Crouch EC, Stenmark KR, Voelkel NF: Smooth muscle-mediated connective tissue remodeling in pulmonary hypertension. *Science* 237:423-426, 1987
- Jacot TA, Striker GE, Stetler-Stevenson MA, Striker LJ: Mesangial cells from transgenic mice with progressive glomerulosclerosis exhibit stable, phenotypic changes including undetectable MMP-9 and increased type IV collagen. *Lab Invest* 75:791-799, 1996
- Pugliese F, Ferrario RG, Ciavolella A, Tamburin M, Benatti L, Casini A, Patrono C, Salvati P: Growth abnormalities in cultured mesangial cells from rats with spontaneous glomerulosclerosis. *Kidney Int* 47:106-113, 1995
- Trevisan R, Yip J, Sarika L, Kuen Li L, Viberti G: Enhanced collagen synthesis in cultured skin fibroblasts from insulin-dependent diabetic patients with nephropathy. *J Am Soc Nephrol* 8:1133-1139, 1997
- Striker GE, He CJ, Liu ZH, Yang DCW, Zalups RK, Esposito C, Striker LJ: Pathogenesis of nonimmune glomerulosclerosis: studies in animals and potential applications to humans. *Lab Invest* 73:569-605, 1995
- Heidland A, Sebekova K, Paczek L, Teschner M, Dammrich J, Gaciong Z: Renal fibrosis: role of impaired proteolysis and potential therapeutic strategies. *Kidney Int* 52 (Suppl. 62):S32-S35, 1997
- MacKay K, Striker LJ, Elliot SJ, Pinkert CA, Brinster RL, Striker GE: Glomerular epithelial, mesangial, and endothelial cell lines from transgenic mice. *Kidney Int* 33:677-684, 1988
- Oliver GW, Leferson JD, Stetler-Stevenson WG, Kleiner DE: Quantitative reverse zymography: analysis of picogram amounts of metalloproteinase inhibitors using gelatinase A and B reverse zymograms. *Anal Biochem* 244:161-166, 1997
- Belkhiiri A, Richards C, Whaley M, McQueen SA, Orr FW: Increased expression of activated matrix metalloproteinase-2 by human endothelial cells after sublethal H<sub>2</sub>O<sub>2</sub> exposure. *Lab Invest* 77:533-539, 1997
- Carome MA, Striker LJ, Peten EP, Elliot SJ, Yang CW, Stetler-Stevenson WG, Reponen P, Tryggvason K, Striker GE: Assessment of 72-kilodalton gelatinase and TIMP-1 gene expression in normal and sclerotic murine glomeruli. *J Am Soc Nephrol* 5:1391-1399, 1994
- Liotta LA, Steeg PS, Stetler-Stevenson WG: Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. *Cell* 64:327-336, 1991
- Matrisian LM: Metalloproteinases and their inhibitors in matrix remodeling. *Trends Genet* 6:121-125, 1990
- Birkedal-Hansen H: Proteolytic remodeling of extracellular matrix. *Curr Opin Cell Biol* 7:728-735, 1995
- Woessner JF: Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB J* 5:2145-2154, 1991
- Conway JG, Wakefield JA, Brown RH, Marron BE, Sekut L, Stimpson SA, McElroy A, Menius JA, Jeffreys JJ, Clark RL, McGeehan GM, Connolly KM: Inhibition of cartilage and bone destruction in adjuvant arthritis in the rat by a matrix metalloproteinase inhibitor. *J Exp Med* 182:449-457, 1995
- Gijbels K, Galdary RE, Steinmann L: Reversal of experimental autoimmune encephalomyelitis with a hydroxamate inhibitor of matrix metalloproteinases. *J Clin Invest* 84:678-685, 1994
- Marti HP, McNeil L, Thomas G, Davies M, Lovett DH: Molecular characterization of a low molecular-mass matrix metalloproteinase secreted by glomerular mesangial cells as Pump-1. *Biochem J* 285:899-905, 1992
- Suzuki D, Miyazaki M, Jinde K, Koji T, Yagame M, Endoh M, Nomoto Y, Sakai H: In situ hybridization studies of matrix metalloproteinase-3, tissue inhibitor of metalloproteinase-1 and type IV collagen in diabetic nephropathy. *Kidney Int* 52:111-119, 1997
- Teschner M, Schaefer RM, Svarnas A, Heidland U, Heidland A: Decreased proteinase activity in isolated glomeruli of streptozotocin diabetic rats. *Am J Nephrol* 9:464-469, 1989
- Reckelhoff JF, Tygart VL, Mitias Walcott JL: STZ-induced diabetes results in decreased activity of glomerular cathepsin and metalloproteinase in rats. *Diabetes* 42:1425-1432, 1993
- Reckelhoff JF, Tygart VL, Racusen LC, Dzielak DJ: Glomerular metalloproteinase activity in streptozotocin-treated rats and in spontaneously diabetic rats (BB/DP). *Life Sci* 55:941-950, 1994
- Marti HP, McNeil L, Davies M, Martin J, Lovett DH: Homology cloning of rat 72 kD type IV collagenase: cytokine and second messenger inducibility in glomerular mesangial cells. *Biochem J* 291:441-446, 1993
- Martin J, Knowlden J, Davies M, Williams JD: Identification and independent regulation of human mesangial cell metalloproteinases. *Kidney Int* 46:877-885, 1994
- Leehey DJ, Song RH, Alavi N, Singh AK: Decreased degradative enzymes in mesangial cells cultured in high glucose media. *Diabetes* 44:929-935, 1995
- Caenazzo C, Garbisa S, Onisto M, Zampieri M, Baggio B, Gambaro G: Effect of glucose and heparin on mesangial  $\alpha$ 1(IV) COLL and MMP-2/TIMP-2 mRNA expression. *Nephrol Dial Transplant* 12:443-448, 1997
- Huhtala P, Chow L, Tryggvason K: Structure of the human type IV collagenase gene. *J Biol Chem* 265:11077-11082, 1990
- Huhtala P, Tuuttila A, Chow L, Lohi J, Keski-Oja J, Tryggvason K: Complete structure for gene of the 92 kD collagenase. *J Biol Chem* 266:16485-16490, 1991
- Matsubara M, Girard MT, Kublin CL, Cintron C, Fini E: Differential roles for two gelatinolytic enzymes of the matrix metalloproteinase family in the

- remodeling cornea. *Dev Biol* 147:425–439, 1991
39. Salo T, Makela M, Kylmaniemi M, Autio-Harmanen H, Larjava H: Expression of matrix metalloproteinase-2 and -9 during early human wound healing. *Lab Invest* 70:176–182, 1994
  40. Canalis E, Rydziel S, Delany AM, Varghese S, Jeffrey JJ: Insulin-like growth factors inhibit interstitial collagenase synthesis in bone cell cultures. *Endocrinology* 136:1348–1354, 1995
  41. Ghazary A, Shen YL, Nedelec B, Wang R, Scott PG, Tredget EE: Collagenase production is lower in post-burn hypertrophic scar fibroblasts than in normal fibroblasts and is reduced by insulin-like growth factor-I. *J Invest Dermatol* 106:476–481, 1996
  42. Flyvbjerg A: Role of growth hormone, insulin-like growth factors (IGFs) and IGF-binding proteins in the renal complications of diabetes. *Kidney Int* 52 (Suppl. 60):S12–S19, 1997
  43. Hirschberg R, Adler S: Insulin-like growth factor system and the kidney: physiology, pathophysiology, and therapeutic implications. *Am J Kidney Dis* 31:901–919, 1998
  44. Flyvbjerg A, Thorlacius-Ussing O, Noeraa R, Ingerslev J, Ørskov H: Kidney tissue somatomedin C and initial renal growth in diabetic and uninephrectomized rats. *Diabetologia* 31:310–314, 1988
  45. Cummings EA, Sochett EB, Dekker MG, Lawson ML, Daneman D: Contribution of growth hormone and IGF-1 to early diabetic nephropathy in type I diabetes. *Diabetes* 47:1341–1346, 1998
  46. Flyvbjerg A, Bornfeldt KE, Marshall SM, Amqvist HJ, Ørskov H: Kidney IGF-1 mRNA in initial renal hypertrophy in experimental diabetes in rats. *Diabetologia* 33:335–338, 1990
  47. Landau D, Chin E, Bondy C, Domene H, Roberts CT Jr, Grønboek H, Flyvbjerg A, LeRoith D: Expression of insulin-like growth factor binding proteins in the rat kidney: effects of long-term diabetes. *Endocrinology* 136:1835–1842, 1995
  48. Flyvbjerg A, Landau D, Domene H, Hernandez L, Grønboek H, LeRoith D: The role of growth hormone, insulin-like growth factors (IGFs) and IGF-binding proteins in experimental diabetic kidney disease. *Metabolism* 44:67–71, 1995