Reversibility of Glucose-Induced Changes in Mesangial Cell Extracellular Matrix Depends on the Genetic Background

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Adequate glycemic control protects most patients with diabetes from nephropathy, but a substantial fraction of patients develop progressive disease despite lowering glycemia. We isolated mesangial cells (MC) from the glomeruli of mouse strains that model these two outcomes in patients with diabetes, namely those that have the propensity (ROP) or resistance (B6) to develop progressive diabetic nephropathy. We determined the nature and reversibility of changes in selected extracellular matrix–related molecules after chronic exposure to elevated glucose concentration. MC were exposed to 25 mmol/l glucose for 5 weeks followed by 6 mmol/l glucose and 19 mmol/l mannitol for an additional 5 weeks. Matrix metalloproteinase-2 (MMP-2) and transforming growth factor-β1 (TGF-β1) levels increased in B6 MC exposed to 25 mmol/l glucose but returned to baseline levels when the glucose concentration was reduced to 6 mmol/l. MMP-2 and TGF-β1 were higher in ROP MC at baseline and increased in response to 25 mmol/l glucose, but remained elevated when glucose concentration was reduced. Type I collagen expression and accumulation increased in a reversible manner in B6 MC exposed to 25 mmol/l glucose. However, type I collagen expression was higher in ROP MC at baseline and remained unaffected by changes in glucose concentration. Thus, 25 mmol/l glucose induced reversible changes in MMP-2, TGF-β1, and type I collagen in MC of sclerosis-resistant mice but not in MC from sclerosis-prone mice. Therefore, progressive diabetic nephropathy may be secondary to stable alterations in the phenotype of MC as a result of the interplay between the genetic background and elevated glucose concentrations. Diabetes 51:499–505, 2002

Nephropathy, a major complication of both type 1 and type 2 diabetes, is the most common single cause of end-stage renal disease in the U.S. (1). Among patients with diabetes, only 20–30% develop progressive nephropathy, and the severity and the rate of progression of the lesions vary widely (1). Improvements in glycemic control reduce the incidence of nephropathy, suggesting that glucose is a major contributor to the development of nephropathy. However, despite tight control of glucose levels, nephropathy appears in a fraction of patients with diabetes (2). Thus, there is a subgroup of patients with diabetes who are prone to the development of nephropathy (sclerosis-prone) and a larger group of patients who are relatively resistant (sclerosis-resistant). We have identified mouse models that mimic the two ends of this spectrum, a sclerosis-prone group that develops rapidly progressing glomerulosclerosis (ROP mice) and a group that is sclerosis-resistant (B6 mice). We have shown that the genetic background is a major determinant of the severity of diabetic glomerulosclerosis in these two models, using streptozotocin (STZ) to induce diabetes (3). The importance of genetic factors in the risk of diabetic nephropathy has also been suggested by studies performed on siblings with diabetes whose glycemic levels were not concordant (4).

Diabetic nephropathy is characterized by an accumulation of extracellular matrix (ECM) components caused by an imbalance between synthesis and degradation (5,6). Mesangial cells (MC) have been postulated to be a major contributor to the sclerotic lesion in glomeruli of patients with diabetes with nephropathy (7). Using nonobese diabetic (NOD) mice, a model of type 1 diabetes with nephropathy, we have shown that isolated MC undergo stable phenotypic changes after the onset of diabetes and that these changes mimic the alterations in ECM turnover found in vivo (8). The changes include modifications in IGF-I synthesis, in MMP synthesis, and in the number of atrial-natriuretic peptide receptors (8,9). Phenotype changes have also been described in skin fibroblasts isolated from patients with diabetes (10–13).

Several experimental models of diabetes confirm the hypothesis that hyperglycemia may be a critical factor in the development of nephropathy in at-risk patients with diabetes. The changes induced by elevated glucose concentration include altered ECM synthesis (14) and degradation (15,16). The induction of transforming growth factor-β1 (TGF-β1) plays a key role in diabetic nephropathy.
factor-β1 (TGF-β1) by elevated glucose concentration is thought to be a major mediator of ECM accumulation (17,18). However, the interplay between genetic susceptibility and glucose concentration in the pathogenesis of diabetic nephropathy has not been investigated. The extent to which hyperglycemic damage may be reversible when normoglycemia is restored has been examined in dogs with diabetic retinopathy (19). In vitro, the reversibility of the effect of chronic exposure to elevated glucose concentration has been examined only in human umbilical vein endothelial cells (20). That changes induced by glucose were not reversible was defined as “glucose memory.”

In the current study, we investigated whether mesangial cells isolated from sclerosis-prone ROP mice and sclerosis-resistant B6 mice differed in their response to chronic exposure to 25 mmol/l glucose and whether the phenotypic changes induced by 25 mmol/l glucose in these two strains were reversible. We examined molecules considered to be relevant to the pathogenesis of diabetic nephropathy, including MMP, TGF-β1, and collagen.

**RESEARCH DESIGN AND METHODS**

**Cell culture and characterization.** MC were isolated from microdissected glomeruli of B6SJLF1/J (B6) and ROP/Lc→EsI1b/Esl1a (ROP) mice. B6 and ROP MC were previously characterized (21,22). Two cell lines for each strain, isolated from different mice, were propagated and used to perform experiments. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM)/F12 (3:1) medium (Gibco BRL) supplemented with 20% fetal bovine serum (FBS; Gibco BRL), 1 mmol/l glutamine (Biofluids), 0.075% NaHCO3, (Gibco BRL), 100 μg/ml penicillin-streptomycin (100 units/ml) (Biofluids), and trace elements (Biofluids). The culture media glucose concentration was 6 mmol/l. MC were propagated in fibronectin-coated Nunc flasks. Phenotypic differences between ROP and B6 MC were studied at both early (3–5) and late passages (15–20) and found to be conserved. For experiments with high ambient glucose, five experimental groups of MC between passages 14 and 20 were examined: 1) MC grown for 10 weeks in 6 mmol/l glucose, 2) MC grown for 10 weeks in 6 mmol/l glucose and 19 mmol/l mannitol, 3) MC grown for 10 weeks in 25 mmol/l glucose, 4) MC grown for 5 weeks in 25 mmol/l glucose and then for 5 additional weeks in 6 mmol/l glucose, and 5) MC grown for 5 weeks in 25 mmol/l glucose and then for 5 additional weeks in 6 mmol/l glucose and 19 mmol/l mannitol. At the end of each experiment, immunocytochemistry for MC markers was performed. In particular, MC stained positively for α-smooth muscle actin (DAKO, Carpinteria, CA) and for smooth muscle myosin (Biomedical Technologies, Stoughton, MA) but negative for CD146 (Chemicon International, Temecula, CA).

**Collagen types I and IV.** MC from each experimental group were plated in six-well plates in media supplemented with 20% FBS in the presence of either 25 mmol/l glucose or 6 mmol/l glucose and 19 mmol/l mannitol. Supernatants and cell layers were collected at a cell density of 60,000/cm2, and enzyme-linked immunosorbent assay (ELISA) was performed as described (23) with the modifications described below. Briefly, standards were plated in the linear range of the curve, ranging between 1.5 and 0.024 ng/μl collagen type I (Collaborative Biomedical Products, Bedford, MA) and between 5 and 0.089 ng/μl collagen type IV (Collaborative Biomedical Products). For the determination of collagen type I, the plates were incubated at 37°C for 2 h, then for 30 min at room temperature in blocking solution (phosphate-buffered saline [PBS]/0.05% Tween-20/0.25% bovine serum albumin), followed by an overnight incubation at 4°C with a rabbit anti-mouse type I collagen diluted 1:2000 (Biodiag International, Kennebunk, ME). A 1:2,000 dilution of a biotinylated goat anti–rabbit IgG antibody was used as secondary antibody (BioSource International, Camarillo, CA) and incubated for 2 h at room temperature. For collagen type IV, samples were similarly processed, but a rabbit anti–mouse type IV collagen antibody diluted 1:3,000 was used (Biodiag International). Final values were expressed as nanogram per 10,000 cells for type I collagen and nanogram per 100,000 cells for type IV collagen.

**MMP activity.** MC were plated as described above for collagen ELISA. Cell number was determined and supernatants were collected at 48 h. For blocking experiments, cells were exposed to either a TGF-β neutralizing antibody (gift from Fibrogen, San Francisco, CA) at the dose of 10 μg/ml for 48 h or an irrelevant isotype-matched antibody (Sigma Chemical, St. Louis, MO) at the same dose. Conditioned media were processed and zymography was performed as previously described (24). The gels were analyzed on a Macintosh computer using the public domain NIH Image program (http://rsb.info.nih.gov/nih-image/). Data from each gel were normalized for average integrated density.

**Reverse transcription–polymerase chain reaction and competitive polymerase chain reaction.** MC were plated into fibronectin-coated 25-cm2 flasks. MC mRNA was extracted with Tri-Reagent (MRC). For blocking experiments, cells were exposed either to a TGF-β neutralizing antibody or to an irrelevant antibody as described above for MMP activity. Reverse transcription and standard polymerase chain reactions (PCRs) were performed as previously described (24).

A mutated cDNA template for MMP-9 was generated for competition studies by PCR using composite primers (5’-CGGTTGATAGGAGGAAAAACCTATTTCCTGCT and 3’-GAGGACTATCTTCTCCTCCGGATAAGAACAC) followed by a second amplification with gene-specific primers (5’-TTCTGCTGACCTCAATGTGG and 3’-GAGATTCTGATCCCGAGGAAGAACAC). PCR with the forward gene-specific and the reverse composite primer yielded two overlapping cDNA fragments. These fragments were mixed in a 1:1 ratio, and the mixture was used as template for a final amplification with the gene-specific primers yielding the mutated cDNA. Product sizes were 414 bp for the wild type cDNA and 366 bp for the mutated cDNA template. Before PCR, all reverse transcription reactions were diluted 1:10 in diethyl pyrocarbonate water; 2 μl of this solution was used as PCR template. Mutated cDNA template for TGF-β1 was previously generated (25). Competitive PCR was performed and analyzed as previously described (26).

**RESULTS**

**Type I and type IV collagen mRNA expression and accumulation.** α1 type I collagen mRNA expression, measured at a cell density of 60,000 cells/cm2, was higher in ROP than in B6 MC at baseline conditions (Fig. 1A). The accumulation of type I collagen protein in the supernatants and cell layers of ROP MC was also higher than in B6 MC (Fig. 1A). B6 MC grown in 25 mmol/l glucose had increased type I collagen mRNA expression and protein levels that were reversible when the glucose concentration was restored to 6 mmol/l (Fig. 1B). Treatment of B6 MC with a TGF-β neutralizing antibody resulted in decreased type I collagen mRNA expression at all glucose concentrations tested, similar to the effect observed in B6 MC (Fig. 1C). No differences in type I collagen mRNA expression were observed between B6 and ROP MC at baseline conditions (Fig. 1A). B6 MC grown in 25 mmol/l glucose had increased type I collagen mRNA expression and protein levels that were reversible when the glucose concentration was restored to 6 mmol/l (Fig. 1B). Treatment of B6 MC with a TGF-β neutralizing antibody resulted in decreased type I collagen mRNA expression at all glucose concentrations tested, similar to the effect observed in B6 MC (Fig. 1C). No differences in type I collagen mRNA expression were observed between B6 and ROP MC at baseline conditions (Fig. 1A). B6 MC grown in 25 mmol/l glucose had increased type I collagen mRNA expression and protein levels that were reversible when the glucose concentration was restored to 6 mmol/l (Fig. 1B). Treatment of B6 MC with a TGF-β neutralizing antibody resulted in decreased type I collagen mRNA expression at all glucose concentrations tested, similar to the effect observed in B6 MC (Fig. 1C).
collagen accumulation were observed between MC exposed to 6 mmol/l glucose alone and MC exposed to 6 mmol/l glucose and 19 mmol/l mannitol in either cell line (data not shown).

Type IV collagen mRNA and protein levels were higher in ROP than B6 MC at baseline conditions (2.2-fold higher mRNA and 1.8-fold higher protein; \( P < 0.05 \)). Changes in the glucose concentration from 6 to 25 mmol/l did not affect type IV collagen mRNA expression (112.8 ± 12.98% of 6 mmol/l in B6 MC and 106.3 ± 18.59% of 6 mmol/l in ROP MC) or protein accumulation (84.63 ± 16.59 vs. 95.69 ± 7.49 ng/100,000 cells in B6 MC and 144.7 ± 19.87 vs. 139.6 ± 25.36 ng/100,000 in ROP MC) in either cell line.

**Pattern of MMP-2 and MMP-9 mRNA expression and enzymatic activity.** We found that ROP and B6 cells had a different pattern of MMP-9 and MMP-2 mRNA expression and activity at baseline. MMP-9 mRNA expression and enzymatic activity in ROP MC were low (nearly undetectable) as compared with B6 MC (Fig. 2A). However, MMP-2 mRNA expression and enzymatic activity were higher in ROP than in B6 MC (Fig. 2B). The levels of MMP-2 and MMP-9 mRNA expression and enzymatic activity were essentially constant to passage 21-22, when cells were cultured in 25 mmol/l glucose, and to passage 25-26 when cells were cultured in 6 mmol/l glucose (data not shown).

MMP-9 mRNA expression by B6 MC was decreased after exposure to 25 mmol/l glucose, and this effect was reversible (Fig. 3A and B). MMP-9 activity was also decreased in B6 MC after exposure to 25 mmol/l glucose (Fig. 3C and D). After restoration of the glucose concentration from 25 to 6 mmol/l, MMP-9 activity returned to 80% of the initial levels in B6 MC. In contrast, we could not detect glucose-induced changes in MMP-9 activity and mRNA expression in ROP MC (data not shown). Osmolarity was not responsible for the decreased MMP-9 activity in B6 MC found in 25 mmol/l glucose, because MMP-9 activity in the MC exposed to 6 mmol/l glucose and 19 mmol/l mannitol was not different from MC exposed to 6 mmol/l glucose alone (data not shown). Similarly, osmolarity was not responsible for the irreversible phenotypic changes observed in ROP MC after restoration of the glucose concentration to 6 mmol/l, because MMP-2 and MMP-9 activity were similar when exposure to 25 mmol/l glucose was followed either by 6 mmol/l glucose or by 6 mmol/l glucose and 19 mmol/l mannitol (data not shown).

MMP-2 mRNA expression (Fig. 4A and B) and activity (Fig. 4C and D) increased after exposure to 25 mmol/l glucose in both ROP and B6 MC. This phenomenon was reversible upon return to 6 mmol/l glucose in B6 MC (\( P < 0.01 \)). However, the levels remained elevated in ROP MC. Treatment with a TGF-β neutralizing antibody did not affect MMP-2 and MMP-9 activity and mRNA expression in
either cell line at any of the glucose concentrations tested (data not shown).

**TGF-β1 mRNA expression.** TGF-β1 mRNA expression at baseline conditions was higher in ROP than in B6 MC (Fig. 5A). Twenty-five mmol/l glucose induced an increase in TGF-β1 mRNA expression in B6 MC, as evaluated by competitive PCR. TGF-β1 mRNA expression was restored to baseline levels after return to 6 mmol/l glucose in B6 MC (Fig. 5B). In contrast, TGF-β1 mRNA expression was increased by exposure to 25 mmol/l glucose in ROP MC, but restoration to 6 mmol/l glucose did not return the levels to baseline (Fig. 5C).

**Response to endogenous and exogenous TGF-β1.** Exogenous administration of human TGF-β1 stimulated the TGF-β–inducible luciferase reporter construct in both cell lines at all glucose concentrations tested (Fig. 6). Pretreatment with a TGF-β neutralizing antibody inhibited the TGF-β–inducible luciferase reporter construct activity of both cell lines exposed to 25 mmol/l glucose and after restoration of the glucose concentration to 6 mmol/l (Fig. 6). Luciferase activity in cells that were treated with either TGF-β1 or TGF-β neutralizing antibody is expressed as fold increase compared with untreated cells within each of the experimental groups (6, 25, or 25/6 mmol/l glucose).

**DISCUSSION**

Although it is well-established that only a fraction of patients with diabetes develop diabetic nephropathy, the mechanisms underlying this disparity are not completely understood. However, it is thought that the genetic background influences both the appearance of diabetic nephropathy and the rate of its progression (4,29). In
addition, glycemic control has been shown to influence the risk of developing diabetic nephropathy (30). We studied the influence of genetic background on the response to chronic exposure to elevated glucose concentration (25 mmol/l) using MC isolated from sclerosis-prone and sclerosis-resistant mice. Furthermore, we determined whether those effects were reversible upon return of the MC from 25 to 6 mmol/l glucose.

We found that MC isolated from sclerosis-prone and from sclerosis-resistant mice had different characteristics at baseline. For instance, baseline MMP-9 expression and enzymatic activity were lower in ROP MC than in B6 MC. In addition, ROP MC had higher MMP-2 mRNA expression and enzymatic activity as well as higher type I collagen and TGF-β1 mRNA expression and protein levels than did B6 MC. These data confirmed and extended our earlier observations that ROP and B6 MC have a different in vitro phenotype (22).

We now report that isolated MC from these two strains differed in the responses of certain ECM-related molecules to alterations in ambient glucose concentrations. Whereas TGF-β1 and MMP-2 levels increased in ROP MC in response to 25 mmol/l glucose, the elevated type I collagen mRNA and protein levels remained unaffected. The absence of changes in type I collagen or MMP-9 by high glucose in ROP MC may reflect the fact that those cells were already maximally stimulated or inhibited at baseline and thus not susceptible to further modulation by increased glucose concentrations. Conversely, when sclerosis-resistant (B6) MC were exposed to 25 mmol/l glucose over a prolonged period of time, we found increases in MMP-2 mRNA and activity, type I collagen mRNA and peptide, and TGF-β1 mRNA. Thus, when sclerosis-resistant MC were chronically exposed to 25 mmol/l glucose, they acquired a phenotype comparable to that of the sclerosis-prone ROP MC at baseline. It is interesting that they also showed decreased MMP-9 mRNA and activity, a characteristic feature of the sclerosis-prone (ROP) MC phenotype. Our results on the MC response to elevated glucose concentrations differ from those reported by others. Namely, while others agreed that MC MMP-9 expression was decreased after exposure to elevated glucose, an increase in MMP-2 has not been consistently observed (15). This may reflect differences in the tissue culture protocol used or in the source of the cells. Indeed, in one study that used human MC and an experimental design similar to ours, MMP-2 activity was always higher in cultures maintained under hyperglycemic than under normoglycemic conditions (16). We did not detect changes in type IV collagen mRNA or accumulation in either cell line after exposure to elevated glucose concentration. This may be related to different experimental conditions and different species. In murine mesangial cells, the stimulation of collagen type IV was maximal after 48 h of exposure to elevated glucose, suggesting that this may be a short-term effect (31). In addition, the accumulation of type IV collagen was not seen in human MC exposed to prolonged hyperglycemia (32), although others reported opposite findings in both rat and human mesangial cells (14,33).

Because the reversibility of changes induced by elevated glucose concentration has obvious clinical relevance, this point was investigated in both cell lines. We found that changes in the expression of certain ECM-related genes induced by 25 mmol/l glucose were reversible in B6 sclerosis-resistant MC but not in ROP sclerosis-prone MC. In particular, MMP-2 mRNA expression and activity and TGF-β1 mRNA expression were induced in both cell lines after exposure to elevated glucose concentrations. However, whereas they were reversible in B6 MC, they remained elevated in ROP MC. The mechanisms underlying the strain-specific differences in reversibility are the subject of current investigations. However, others have postulated that there is a phenomenon called “glucose memory” (20). This has been attributed to the ability of glucose to induce stable mitochondrial DNA mutations (34). It is possible that susceptible individuals have an increased instability of DNA in regions that influence the expression of genes implicated in glomerulosclerosis, similar to what is described for the susceptibility to develop cancer (35).

We then examined the role of TGF-β1 in glucose-mediated regulation of ECM. Interestingly, whereas type I collagen was regulated by TGF-β1, this was not the case for MMPs. This was shown by experiments that demonstrated that the presence of a TGF-β neutralizing antibody...
did not alter MMP-2 and MMP-9 mRNA expression and enzymatic activity. TGF-β has been shown to act through the promoter region of several MMPs, which contain either a TGF-β inhibitory element (TRI) or AP-1 sites (15). Our finding may be explained by the absence of TRI sites in the promoter regions of MMP-2 and MMP-9, together with the absence of an AP-1 site in the MMP-2 promoter. The increased type I collagen mRNA levels and peptide accumulation observed in B6 MC after exposure to elevated glucose concentrations was mediated through TGF-β1, as previously described (36). Although elevated glucose concentrations did not lead to additional increases in type I collagen in ROP MC, baseline type I collagen mRNA expression was blocked by pretreatment with a TGF-β neutralizing antibody. This suggests that the TGF-β1 system might already be maximally upregulated in ROP MC at baseline. Finally, we showed that the irreversibility of the changes induced by high glucose concentration in ROP MC was not due to an altered response to TGF-β1. The TGF-β pathway was demonstrated to be intact in both B6 and ROP MC when exposed to high glucose. Thus, although there are strain-specific differences at baseline and in response to elevated glucose levels, manipulation of the TGF-β1 pathway may still represent a valid therapeutic approach in the treatment of the susceptible individuals. In conclusion, these data suggest that mesangial cells from diabetic mice that progress to nephropathy are characterized by a sclerosing phenotype. This altered phenotype is manifested by failure of ECM molecules to return to baseline values when chronically elevated glucose levels were normalized, whereas MC from mice that do not develop glomerulosclerosis return to baseline levels when glucose levels are lowered. The latter finding stresses the importance of good glycemic control in a subgroup of patients with diabetes whose MC phenotype might resemble that of the sclerosis-resistant mice.

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REFERENCES


