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Expression of matrix metalloproteinases MMP-2 and MMP-9 is altered during nephrogenesis in fetuses from diabetic rats

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Remodeling of extracellular matrix (ECM) is an important physiological feature of normal growth and development. Recent studies have emphasized the role of matrix metalloproteinases (MMP-2 and MMP-9) in normal mouse nephrogenesis. We have demonstrated previously in the rat that *in utero* exposure to maternal diabetes impairs renal development leading to a 30% reduction in the nephron number. Transforming growth factor-β1 (TGF-β1) and connective tissue growth factor (CTGF) are known to mediate high glucose effects on matrix degradation. The aim of the present study was to address the expression of type IV collagenase and TGF-β1/CTGF systems in rat kidney during normal development and after *in utero* exposure to maternal diabetes. Both MMP-2 and MMP-9 mRNA metanephric expressions and activities were dramatically downregulated in kidneys issued from diabetic fetuses and in metanephros cultured in the presence of high glucose concentration. TGF-β1 and CTGF expressions were significantly enhanced in diabetic fetal kidneys and in high glucose cultured metanephroi. Conditioned media obtained from metanephroi grown with high glucose concentration upregulated functional TGF-β activity in transfected ATDC5 cells. In conclusion, in impaired nephrogenesis resulting from *in utero* exposure to maternal diabetes, alteration of both type IV collagenase and TGF-β1/CTGF systems may lead to abnormal remodeling of ECM, which may, in turn, induce defects in ureteral bud branching leading to the observed reduction in the nephron number with consequences later in life: progression of chronic renal disease and hypertension.

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The development of the kidney is based on reciprocal induction between two mesodermally derivated tissues. Morphogenesis starts when the ureteric bud (UB) invades the metanephric mesenchyme and initiates reciprocal induction. The mesenchyme induces the UB to grow and branch to form the collecting ducts. The UB, in turn, induces the mesenchyme to undergo a mesenchymal to epithelial conversion to form nephrons via a series of morphogenetic events (aggregation of mesenchyme to form renal vesicles then S-shaped bodies and finally mature glomeruli).[1,2] Regulation of this process involves the participation of various DNA-binding proteins, that is, transcription factors, growth factors and their receptors, and proto-oncogenes acting as growth factor receptors. A constant remodeling of the extracellular matrix (ECM) is needed at the growing tips of the invading uretric bud to allow further branchings in the metanephric mesenchyme.[3,4] Production of ECM-degrading enzymes is expectedly linked to metanephric development to regulate the expression of ECM protein and ECM-related proteins by proteolytic processing. This creates a gradient of all these molecules in strategic locations with spatiotemporal distribution.[4] The ECM-degrading enzymes are collectively known as matrix metalloproteinases (MMPs) because their activity is dependent on the presence of metal ion, for example, Zn^{2+}. The activity of MMPs is inhibited by tissue inhibitors of MMPs (TIMP-1–4). Among the MMPs, type IV collagenase MMP-2 and MMP-9 have been studied in normal nephrogenesis, mainly in the mouse and with conflicting results.[4–12]

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Diabetes is associated with a high incidence of congenital malformations including urogenital abnormalities.[13,14] These congenital malformations result from developmental defects occurring in early organogenesis. They include genito-urinary abnormalities that can be so severe as to cause renal agenesis.[15] Using both streptozotocin (STZ)-induced diabetes and glucose infusion rat models, we have demonstrated that *in utero* exposure to hyperglycemia impairs nephrogenesis in fetuses.[16] The previous study identifies maternal diabetes as a novel risk factor for inborn nephron deficit. According to recent works, a congenital nephron deficit, even moderate, is a risk factor for progression in patients with chronic renal disease and hypertension in adulthood.[17–19]

Increased deposition of ECM is the hallmark of diabetic complications in the adult kidney:[20] diabetic glomerulosclerosis is characterized by accumulation of mesangial matrix that has been linked to a reduction in MMP activities (mainly MMP-2 activity).[21–24] An effect of glucose on the *in vitro* regulation of type IV collagenase system has been clearly demonstrated in various cell systems.[21,23,24] Recent studies have demonstrated the crucial role of both transforming growth factor-β1 (TGF-β1) and its downstream mediator, connective tissue growth factor (CTGF) in the glucose-induced dysregulation of MMP in experimental diabetic nephropathy.[21,23–28] Little is known concerning the implication of MMP and TGF-β1/CTGF systems in diabetic nephrogenesis. Several studies have reported that *in vitro* exposure of metanephroi to high glucose concentration causes alteration in extracellular components which may explain the dysmorphogenesis of the embryonic kidney.[29,30] On the other hand, these authors have also shown that maternal diabetes increases the synthesis of ECM components in developing embryos.[3] Altogether, these studies strongly suggest that maternal diabetes may induce an alteration of nephrogenesis via glucose-impaired ECM regulation.

Thus, the present study was performed to investigate the expression of type IV collagenase system and TGF-β1/CTGF system in rat metanephros during both normal and diabetic-altered nephrogenesis and in metanephros cultured in presence of high glucose concentration.

Our results show that type IV collagenase system is developmentally regulated in normal nephrogenesis and that its expression is altered during diabetic kidney development. This alteration is associated to an enhancement of TGF-β1/CTGF system.

MATERIALS AND METHODS

Experimental Protocol

Female Sprague–Dawley rats given free access to water and standard laboratory pellets (UAR Laboratory, Villemoison sur Orge, France), were caged overnight with a male and vaginal smears were taken the following morning. The day a positive smear was obtained was designated as day 0 of gestation. Pregnant females were made diabetic, as described previously, by a single injection of 40 mg/kg body weight of STZ (Sigma, Saint Quentin Fallavier, France).[16] The experiments were performed in fetuses removed from anesthetized pregnant female rats on days 14, 16, 18, and 20 of gestation. Metanephric kidneys were surgically removed from fetuses and were fixed in 4% formalin for histological and *in situ* hybridization studies. The experiments were conducted in accordance with the institutional guidelines and the recommendations for the care and use of laboratory animals put forward by the French Ministry of Agriculture.

Table 1 Primer list and characteristics

		Primers	Fragment size (bp)	Annealing temperature (°C)
β-actin	Sense	5′-GGG ACG ATA TGG AGA AGA TTT	315	61.1
	Antisense	5′-GCT GGG GTG TTC GAG GT		
MMP-2	Sense	5′-CCT GAA TAC TTT CTA TGG CTG C	631	61.3
	Antisense	5′-GTA TGT AGT GGA GCA CCA GAG C		
MMP-9	Sense	5′-GCA ACG GAG ACG GCA AAC C	170	57.9
	Antisense	5′-GAC GAA GGG GAA GAC GCA		
TIMP-1	Sense	5′-CAT CGA GAC CAC CTT AT	193	60.4
	Antisense	5′-CAC CCC ACA GCC AGC ACT ATA		
TIMP-2	Sense	5′-CAA AGC AGT GAG CGA GAA GG	393	61.6
	Antisense	5′-ACC CAG TCC ATC CAG AGG GA		
TGF-β1	Sense	5′-GAA CCA AGG AGA CGG AAT ACA G	248	60
	Antisense	5′-TCT CTG TGG AGC TGA AGC AGT A		
CTGF	Sense	5′-TGT ATG GAG ACA TGG CGT AAA G	189	60
	Antisense	5′-ATG ACA ATG ACA CAC GGT TCT C		

Histology and Immature (S-Shaped Bodies) and Mature Glomeruli Counts

Longitudinal frontal renal cross sections were taken through the hilus and embedded in paraffin. A total of 4-μm-thick serial sections were cut and stained with hematein and eosin for morphological analysis. The total numbers of immature (S-shaped bodies) and mature glomeruli were determined in kidneys of fetuses at gestational day 18 on whole longitudinal sections taken through the hilus, assuming the results would be representative of the number of nephrons within the entire kidney.[31] The initial stages of glomerular development (renal vesicles) were excluded from this counting. Glomeruli were considered as mature when they clearly demonstrated both distinct Bowman's spaces and vascularized floculi, regardless of the glomerular width. The mean number of immature and mature glomeruli was counted one by one, by scanning the entire kidney sections (in respectively six and eight kidneys from control and diabetic mothers) by three different investigators who were unaware of the specimen groups.

Metanephric Organ Culture

Embryonic rat kidneys were microdissected at 14 days of gestation and cultured as described previously.[32] Briefly, the metanephroi were placed on a 0.8 μM polycarbonate filter (Millipore, Saint-Quentin-en-Yveline, France) flotting on a defined serum free medium and incubated for 6 days in 35 mm Petri dishes at 37°C in a humidified incubator containing 5% CO_2. One metanephros from each fetus was grown in control medium, and the controlateral metanephros was grown in the same medium with glucose added (14 mM). All media were changed daily. Nephrons formed *in vitro* were visualized after labeling the explanted metanephroi as described previously[16] using specific lectins, the helix pomatia agglutinin coupled to fluorescein, which stains tubules and glomeruli and peanut agglutinin (PNA) coupled to rhodamine, which stains only glomeruli.

Quantitative RT-PCR

Quantitative RT-PCR was performed as described previously.[33] Total RNA was isolated from the kidneys of different embryonic days with Tri-reagent (Invitrogen). Before reverse transcription, all RNA samples (2 μg) were freed of any contaminating DNA by treatment with a RNase-free DNase I according to the manufacturer protocol (Invitrogen). First-strand cDNA synthesis was primed by Superscipt II reverse transcriptase (200 U) with 250 ng random primers. Negative control tubes were performed to ensure the absence of DNA contamination. Resulting single stranded cDNA was used as a starting material for quantitative assay of the transcript by real-time PCR. Primer pairs used for cDNA amplifications are listed in Table 1. The quality and specificity of the resulting amplified PCR fragment were also carefully checked. Quantitative PCR experiments, carried out to determine mRNA concentrations, were performed on a Light Cycler apparatus (Roche Diagnostics). SYBR Green dye was used as a reporter for detection according to protocols from Roche Diagnostics. Reactions were conducted in glass capillaries, subjected to a 10-min initial hot-start activation of the Taq polymerase at 95°C, followed by an adapted cycle numbers of amplification (Table 1).

Final data were expressed as the ratio between the amount of MMP-2, MMP-9, TIMP-1, TIMP-2, TGF-β1, and CTGF transcripts and the amount of β-actin transcript. All calculations were made with the Light Cycler software (version 3, Roche Diagnostics). After running the experiments, two types of quality controls were carried out. First, final products were submitted to a Light Cycler built-in renaturing–denaturing thermal program. Fusion curves obtained during this step gave confirmation of the homogeneity of the amplified fragments. Second, all the amplified products were electrophoresed on an agarose gel for visual inspection.

In Situ Hybridization

In situ hybridization was performed as described previously.[34] Following fixation in formalin, tissues were embedded in

Table 2 Maternal blood glucose concentration and insulinemia, number of fetuses and mean fetal body weight on gestational days 14, 16, 18, and 20

Days of gestation	Groups	Maternal blood glucose concentration (mmol/l)	Maternal insulinemia (pmol/l)	Number of fetuses by litter	Mean fetal body weight (mg)
14	Control ($n=16$)	5.5 ± 0.6	172.7 ± 2.4	14.3 ± 1.4	161.0 ± 6.9
	STZ ($n=14$)	23.1 ± 3.2****	122.1 ± 3.2****	13.5 ± 2.1	144.9 ± 7.8*
16	Control ($n=3$)	5.7 ± 0.5	175.2 ± 4.1	11.7 ± 2.5	458.3 ± 6.0
	STZ ($n=3$)	22.7 ± 3.1***	130.1 ± 1.3***	14.0 ± 2	407 ± 8.9*
18	Control ($n=5$)	5.3 ± 0.7	174.6 ± 4.1	15.6 ± 3.3	1420 ± 50
	STZ ($n=3$)	22.8 ± 1.7***	126.1 ± 4.2***	15 ± 1.7	1270 ± 70*
20	Control ($n=3$)	5.3 ± 0.7	175.1 ± 4.1	10.7 ± 3.2	3640 ± 90
	STZ ($n=3$)	22.3 ± 2.6***	128.2 ± 2.3***	12.7 ± 4.7	3380 ± 150*

*$P<0.05$, ***$P<0.001$, ****$P<0.0001$ compared to control.

paraffin. Deparaffinized 5-μm-thick sections were digested for 15 min at 37°C with 15 μg/ml protease K (Sigma) in a buffer containing 20 mM Tris-HCl, pH 7.5, and 2 mM $CaCl_2$. MMP-2 and MMP-9 cDNA probes were labeled with ^{35}S dCTP using Megaprime DNA labeling system (Amersham, Les Ulis, France). Hybridization with ^{35}S-labeled rat probes (0.2 μg/ml) was performed overnight at 45°C in 40% formamide, 4 × SSC, 10% dextran, 1 × Denhardt, 0.8 mg/ml yeast tRNAs, and 0.5 mg/ml denatured salmon sperm DNA. Sections were washed two times at 45°C for 15 min in 40% formamide and 4 × SSC, once at 60°C for 30 min in 4 × SSC, and three times at room temperature for 20 min in 2 × SSC. After dehydration, sections were dipped into NTBII autoradiographic emulsion (Kodak, Rochester, NY, USA) and exposed at 4°C for 21 days. Slides were finally immersed in D19 developer (Kodak), fixed in A44 Kodak and counterstained with hematoxylin and eosin. Control slides (50 μg/ml) were treated by RNase A (Sigma) at 37°C for 30 min in 2 × SSC and washed at room temperature for 15 min in 2 × SSC before the hybridization.

Substrate Gel Electrophoresis (Zymography)

In kidneys from fetuses of 14, 16, 18, and 20 days day post coitus (dpc) issued from control and diabetic mothers, metalloproteinases were detected and characterized by zymography as described previously.[9] The gelatinolytic activity of MMP-2 and MMP-9 was demonstrated by zymography. Kidney proteins were solubilized in lysis buffer (50 mM Tris, 150 mM NaCl, NP-40 1%, 10 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, pH 8) overnight at 4°C. About 20 μg of protein, measured by a Bio-Rad protein assay (Bio-Rad Laboratories, München, Germany) was loaded on 8% SDS-polyacrylamide (Merck, Darmstadt, Germany) and the gels were co-polymerized with 1 mg/ml of gelatin or type IV collagen (Sigma). Electrophoresis was performed under non-reducing conditions at 25 mA. Gels were washed two times for 30 min in 2.5% Triton X-100 to remove SDS, incubated in substrate buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.01% NaN_3, pH 7.5) overnight at 37°C, stained in 2.5% Coomassie Blue G (Sigma-Aldrich, St Louis, MI, USA) prepared in 50% methanol, 10% acetic acid for 30 min at room temperature and destained three times for 15 min in buffer composed of 30% methanol and 1% acetic acid. The presence of metalloproteinases was indicated by an unstained proteolytic zone of the substrate. The identity of MMP-2 and MMP-9 was assessed by their respective molecular weight and by using conditioned media from principal cell line.[35]

Functional Activity of TGF-β

Functional activity of TGF-β1 was performed as described previously.[36] ATDC5 cells in a six-well plates (10^5 cells per well) were transfected in serum-free medium using FuGENE (Roche) as described in the user's manual. For reporter assays, 0.5 μg of TGF-β specific responsive promoter (CAGA) 9-MLP-Luc, (a gift from JM Gauthier), was co-transfected with 0.1 μg of pCMV β-galactosidase as an internal transfection control (Clontech). After 24 h of transfection, serum-starved cells were treated for an additional 24 h period with conditioned medium, obtained from metanephroi cultured with or without glucose. Conditioned medium from metanephric organ culture was acidified according to McCormick *et al*[37] to reveal TGF-β activities. Luciferase and β-galactosidase activities were assayed using kits from

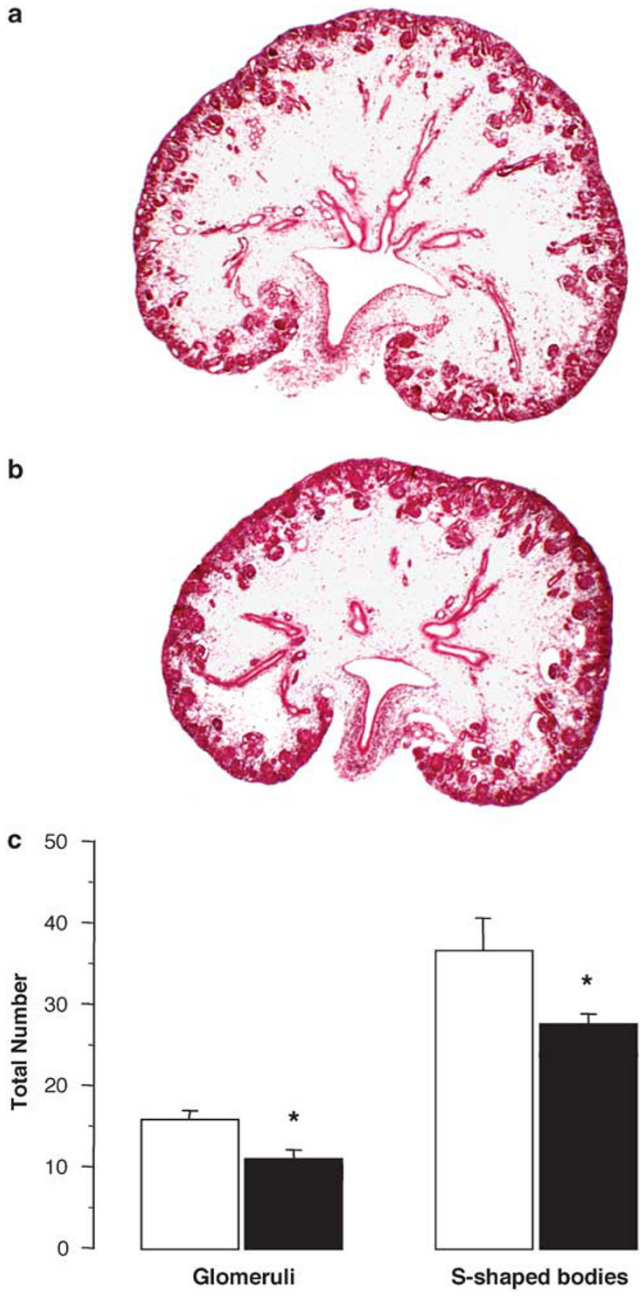

Figure 1 Representative kidneys of fetuses from control (**a**) and diabetic (**b**) rats on day 18 of gestation. Hematein and eosin. Panel c illustrated means of S-shaped bodies and glomeruli counts on kidney tissue sections from control (open bars, $n = 6$) and diabetic (black bars, $n = 8$) rats. Values are means ± s.e.m. $^*P < 0.05$.

Promega and Perkin Elmer Life Sciences (Galacto-Star system). For each experiment performed on two separate occasions, conditioned media from three different metanephroi were pooled and the incubation was performed in triplicate.

Statistical Analysis

Data are presented as means $\pm$ s.e.m. Differences between control and hyperglycemic groups were compared by Student's unpaired *t*-test, except in the case of data from metanephric organ cultures in which paired *t*-tests were used. The threshold of significance was taken to be $P<0.05$.

RESULTS

STZ-Induced Diabetic Rats and Histological Assessment of Diabetic Impaired Nephrogenesis

As shown in Table 2, the mean maternal plasma glucose and insulin concentrations in STZ group remained constant and was about three times higher for glucose (STZ group: 22.9 ± 0.1 mmol/l, $n=23$ *vs* control group: 5.5 ± 0.1 mmol/l, $n=27$); and about 30% lower for insulinemia (STZ group: 124.9 ± 1.3 mmol/l, $n=23$ *vs* control group: 173.6 ± 1.7 pmol/l, $n=27$), respectively. Gestation occurred normally. The number of fetuses by litter was similar in the two groups. The mean body weights of fetuses from STZ mothers were reduced by about 10% as compared to controls. As illustrated in Figure 1, we confirmed the alteration of nephrogenesis reported previously using this animal model in 18-day-old fetuses from STZ mothers of the present study.[16] Kidneys from STZ fetuses (panel b) were slightly smaller than their normal counterpart (panel a). The number of immature (S-shaped bodies) and mature glomeruli per kidney section was significantly lower in the STZ fetuses (S-shaped bodies: 26.3 ± 1.3 in STZ ($n=5$) *vs* 35.8 ± 4.0 $P<0.05$ in control ($n=8$); mature glomeruli: 10 ± 1.1 in STZ ($n=5$) *vs* 15.9 ± 1.1 in control ($n=8$), $P<0.05$).

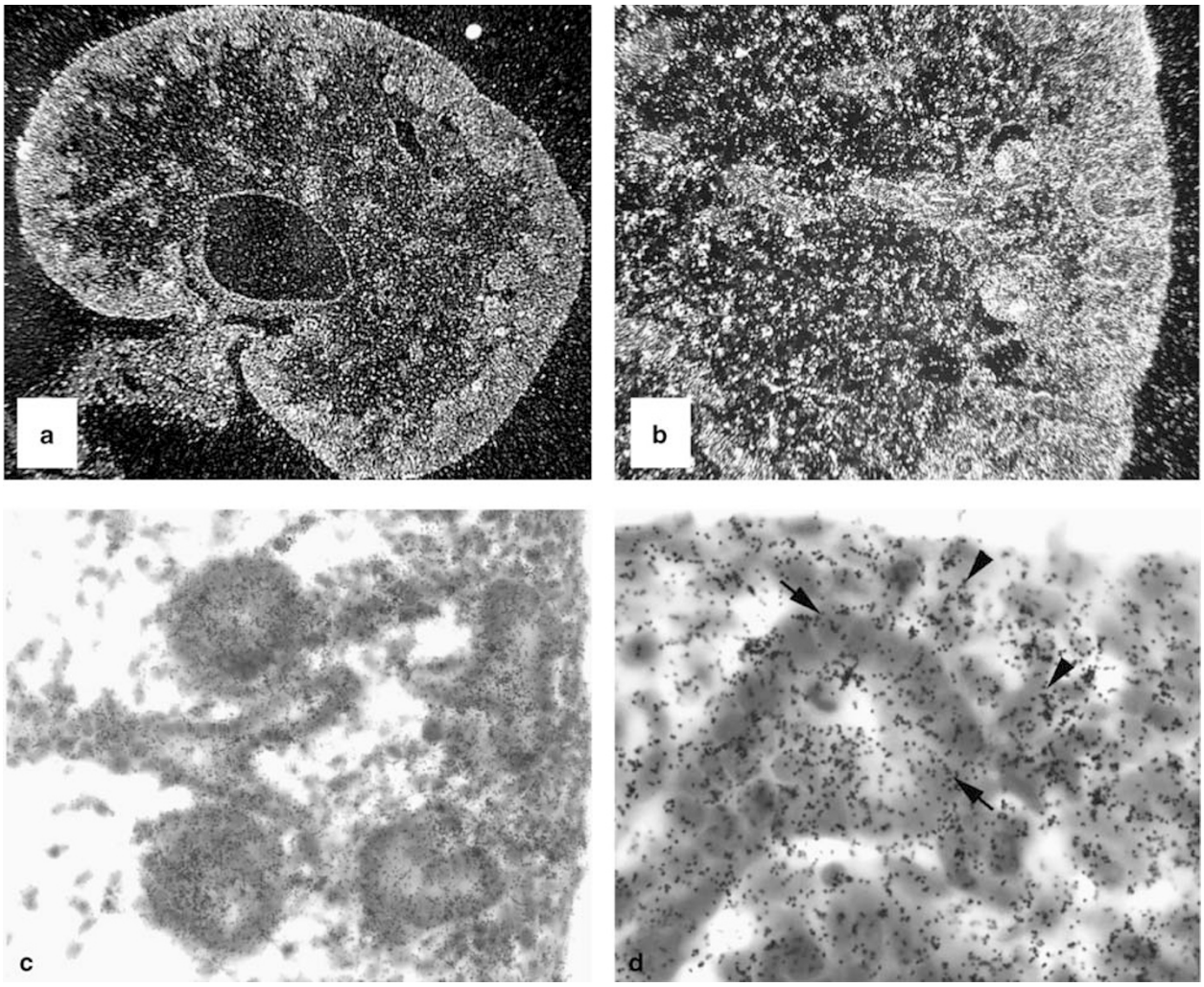

Figure 2 Representative *in situ* hybridization with rat MMP-2 ^{35}S-labeled cDNA probe in fetal kidneys from control rats on day 18 of gestation. Hybridization signal is mainly present in the cortex (**a** and **b**), where both epithelial and mesenchymal structures are labeled (**c**). In the nephrogenic zone (**d**), a strong signal was observed in both UB (arrows) and condensated mesenchyme (arrowheads).

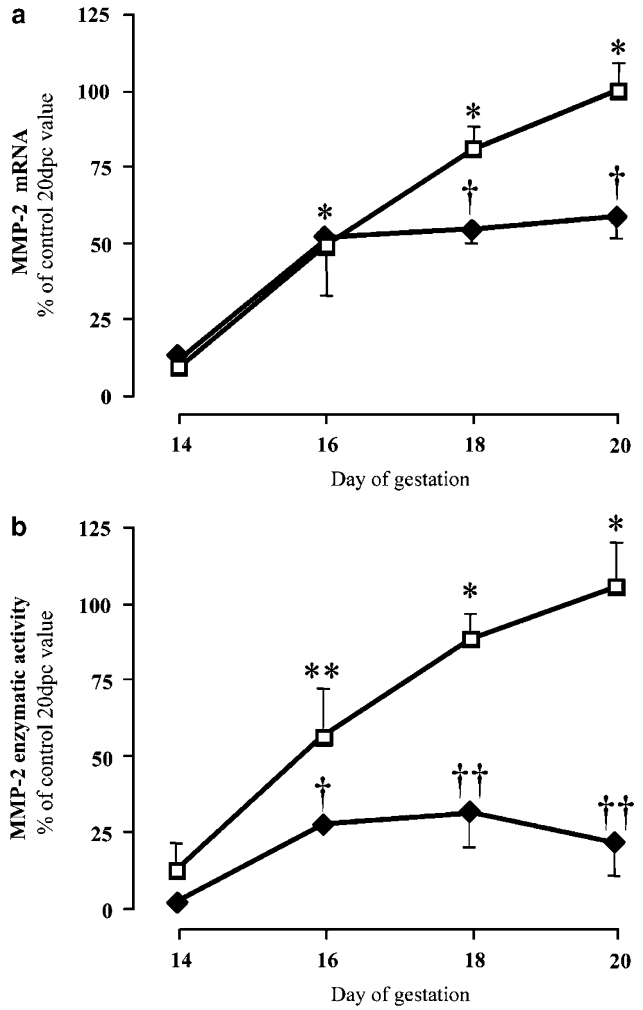

Figure 3 Analysis of MMP-2 during normal and diabetic kidney development. Expression pattern (**a**) and enzymatic activity (**b**) of MMP-2 in control (open squares) and diabetic (black diamonds) kidneys (14–20 dpc). Values are expressed as a percentage of 20 dpc control values and represent the mean of four experiments. $^{*}P<0.05$ and $^{**}P<0.01$ compared with the precedent stage and $^{\dagger}P<0.05$ and $^{\dagger\dagger}P<0.01$ compared with age-matched control fetuses.

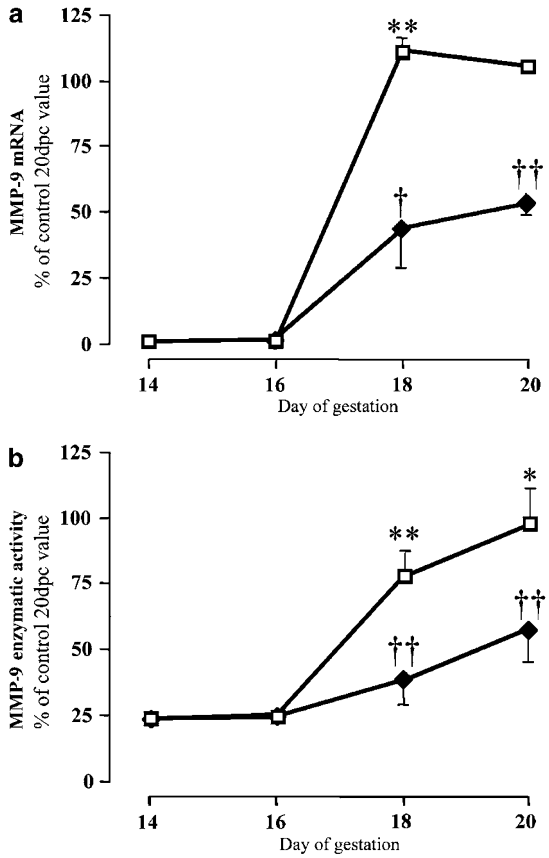

Figure 4 Analysis of MMP-9 during normal and diabetic kidney development. Expression pattern (**a**) and enzymatic activity (**b**) of MMP-9 in control (open squares) and diabetic (black diamonds) kidneys (14–20 dpc). Values are expressed as a percentage of 20 dpc control values and represent the mean of four experiments. $^{*}P<0.05$ and $^{**}P<0.01$ compared with the precedent stage and $^{\dagger}P<0.05$ and $^{\dagger\dagger}P<0.01$ as compared with age-matched control fetuses.

Altered Expression of Type IV Collagenase System during Diabetic-Altered Nephrogenesis

In control fetal rat kidney (gestational day 18), *in situ* hybridization (Figure 2) shows that MMP-2 mRNA expression was mainly present in the nephrogenic zone with a strong expression in the epithelial structure, the UB and the condensed mesenchyme. Despite several attempts, we failed, with our labeled probe specific of MMP-9 mRNA, to localize the expression of MMP-9 in the fetal kidney (data not shown). This may be probably due to the very low level of MMP-9 mRNA present in fetal metanephroi.

Figures 3–5 depict the modulation of type IV collagenase system expression in 14–20 dpc kidneys from control (open squares) and STZ fetuses (black diamonds).

During normal rat nephrogenesis (Figure 4, open squares) MMP-2 and MMP-9 mRNA and functional activities are strongly increased. In kidneys of STZ fetuses a significant reduction is observed: MMP-2 and MMP-9 expressions were reduced by 50% at 20 dpc as compared to control values. Currently, MMP-2 enzymatic activity collapsed, representing less than one-fifth of control value. Localization of MMP-2 mRNA expression in kidneys of STZ fetuses was similar to that observed in controls (data not shown).

mRNA expressions of TIMP-2 and TIMP-1, MMP-2 and MMP-9 inhibitors, respectively, were unchanged between control and diabetic groups (Figure 5). The expression of TIMP-2 was constant during nephrogenesis while that of TIMP-1 was increased.

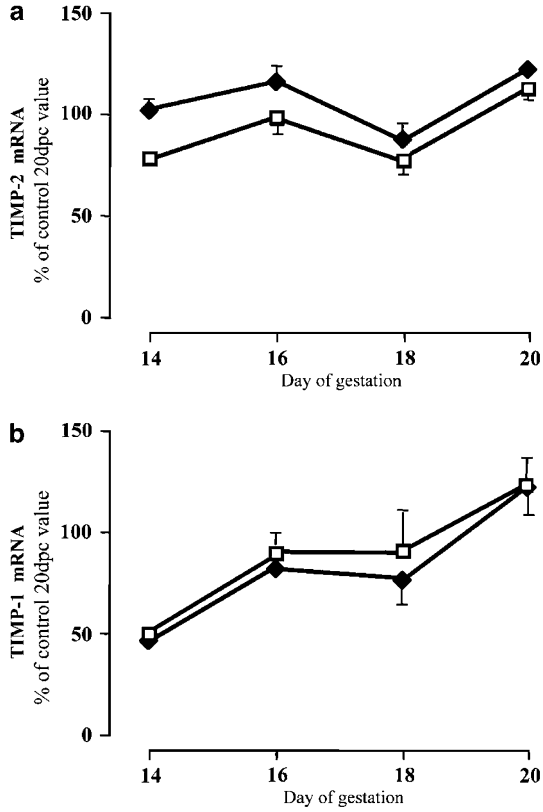

Figure 5 Analysis of TIMP-2 and TIMP-1 during normal and diabetic kidney development. Expression pattern (14–20 dpc) of TIMP-2 (**a**) and TIMP-1 (**b**) in control (open squares) and diabetic (black diamonds) kidneys. Values are expressed as a percentage of 20 dpc control values and represent the mean of four experiments. $^{*}P<0.05$ and $^{**}P<0.01$ compared with the precedent stage; and $^{\dagger}P<0.05$ and $^{\dagger\dagger}P<0.01$ compared with age-matched control fetuses.

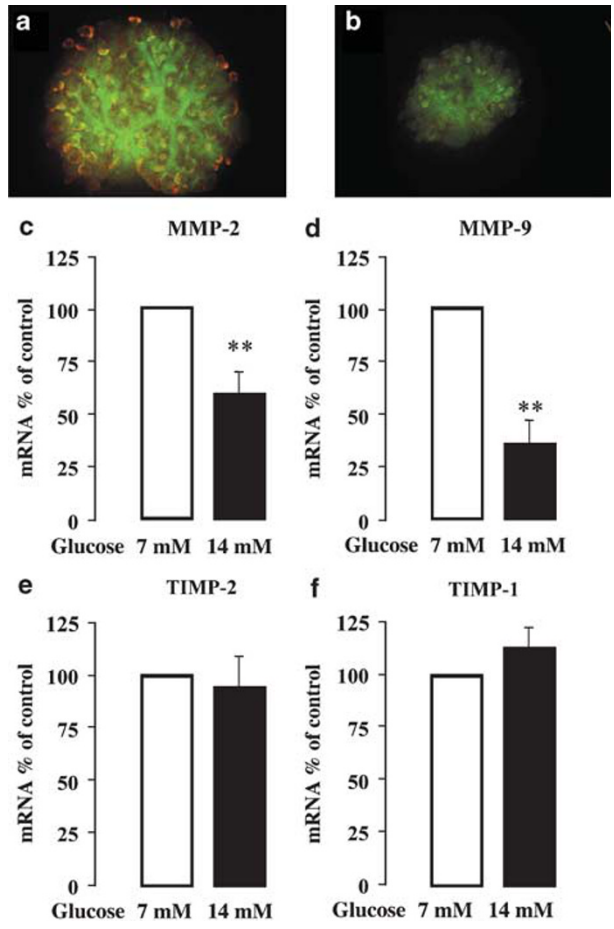

Figure 6 Analysis of type IV collagenase system during metanephroi development *in vitro*. Explanted metanephroi from 14-day-old embryos were cultured with 7 mM (**a** and open bars) or 14 mM (**b** and black bars) glucose. Metanephroi development was assessed by lectin histochemistry, using PNA and dolichos biflorus agglutinin labeling (**a** and **b**). The expression of MMP-2 (**c**), MMP-9 (**d**), TIMP-2 (**e**) and, TIMP-1 (**f**) in metanephric explants cultivated with 7 mM (open bars) or 14 mM (black bars) glucose were assessed by quantitative RT-PCR. Histograms represent quantification of mRNA expressed as a percentage of control values (means $\pm$ s.e.m. from four independent experiments). $^{**}P<0.05$ as compared to control value.

Decreased Expression of Type IV Collagenase System in Metanephric Organ Culture in Response to High Glucose Concentration

Addition of glucose at high concentration in culture medium (14 mM) strongly impaired *in vitro* metanephric growth (Figure 6). Both MMP-2 and MMP-9 mRNA levels were significantly reduced in this latter condition while expression of TIMP-2 and TIMP-1 were similar in both culture conditions.

Hyperglycemia Enhances the Expression of Both TGF-β1 and CTGF in Fetal Kidney

A new set of experiments was then designed to address the involvement of TGF-β1 and CTGF in diabetic nephrogenesis. Because the role of TGF-β1 has been clearly demonstrated in UB division, we assessed—in metanephroi obtained from control and STZ fetuses—the expression of TGF-β1 and CTGF at 16 dpc, a stage when UB branching is prominent. Results show that at this stage, maternal hyperglycemia strongly upregulated the expression of both TGF-β1 and CTGF (Figure 7a), in parallel with the decreased activity of MMP-2 but without alteration of MMP-9 activity (see Figures 2 and 4). We then studied in cultured metanephroi the expression of TGF-β1 and CTGF *in* vitro (panel b). In high glucose concentration medium, quantitative RT-PCR showed a strong enhancement of TGF-β1 and CTGF expression. Finally, to assess whether the TGF-β1 upregulated mRNA expression could result in higher TGF-β potential activity under these culture conditions, we used a promoter reporter bioassay. Compared to the control, the conditioned medium obtained from metanephros cultured with high glucose concentration indeed increased to a higher extent the luciferase activity driven by a TGF-β-specific responsive promoter (Figure 7c).

DISCUSSION

The present study describes the expression of type IV collagenase and TGF-β1/CTGF systems during rat normal and diabetic-altered nephrogenesis.

Our results show that the expression and functional activity of MMP-2, MMP-9, and TIMP-1 and TIMP-2 inhibitors are regulated in normal rat nephrogenesis and that MMP-2 and MMP-9 expressions are altered during kidney development in STZ fetuses. Both TGF-β1 and CTGF metanephric expressions are also significantly enhanced *in vivo* by exposure to maternal diabetes and *in vitro* in the presence of high concentration of glucose in organotypic culture.

Although the role of ECM-degrading enzymes in organotypical epithelial–mesenchymal interaction was originally proposed more than three decades ago,[38,39] recent evidence concerning their implication in nephrogenesis has only been found within the last few years, with conflicting results. Our results strongly support the crucial role of type IV collagenase system during nephrogenesis in the rat suggested by others in mice and in *in vitro* models.[4–12] We found that both MMP-2 and MMP-9 expression and activity rise in the fetal kidney throughout normal gestation. Similar results have been reported in mice.[7] *In vitro* their levels decrease during the postnatal period, suggesting that their potential role in the organogenesis of the kidney is confined to the embryonic period.[9] MMP-2 expression in normal rat metanephros increases continuously while MMP-9 expression, nearly undetectable at 14 and 16 dpc, strongly increases in late nephrogenesis. We also show that TIMP-2 expression is present throughout the whole nephrogenesis and at high levels since 14 dpc. This result is in accordance with the presence of TIMP-2 in cell lines derived from 13 dpc rat metanephros.[6] TIMP-1 expression during nephrogenesis follows MMP-9 levels.

In situ hybridization in 18 dpc rat metanephroi shows that MMP-2 mRNA expression is mainly present in the nephrogenic zone in the epithelial structures (UB) and in the condensed mesenchyme, a structure that undergoes an epithelial conversion to form nephrons. This pattern of distribution strongly suggests that this system is involved in epithelial–mesenchymal interactions.[10] Sakurai and Nigam[40] and Sakurai *et al*[41] reported a parallel increase (or fall) in the MMP-2 mRNA expression with stimulation (or inhibition) of branching morphogenesis under the influence of TGF-β or of epidermal growth factor receptor ligands. Furthermore, Pohl *et al*[11] have recently reported that metanephric mesenchyme cell-derived conditioned medium alters the transcription of MMP-2, MMP-3, MMP-9, MT1-MMP, and TIMP-2 along with the changes in UB branching morphogenesis. Moreover, treatment of mouse metanephric explants by MMP-2 and MT-1-MMP antisense oligodeoxynucleotides results in the reduction of embryonic metanephros size and in dysmorphogenic changes of the UB, both effects that were partially reversed by co-incubation with TIMP-2 antisense oligonucleotides.[7] MMP-9, expressed in the metanephros, has been shown to play a role in the organogenesis of the kidney *in vitro*.[9] Here, we were unable to localize MMP-9 transcript, probably because of its very low level of expression as illustrated by quantitative RT-PCR experiments in which a high number of cycles were needed to obtain a signicative amplification.

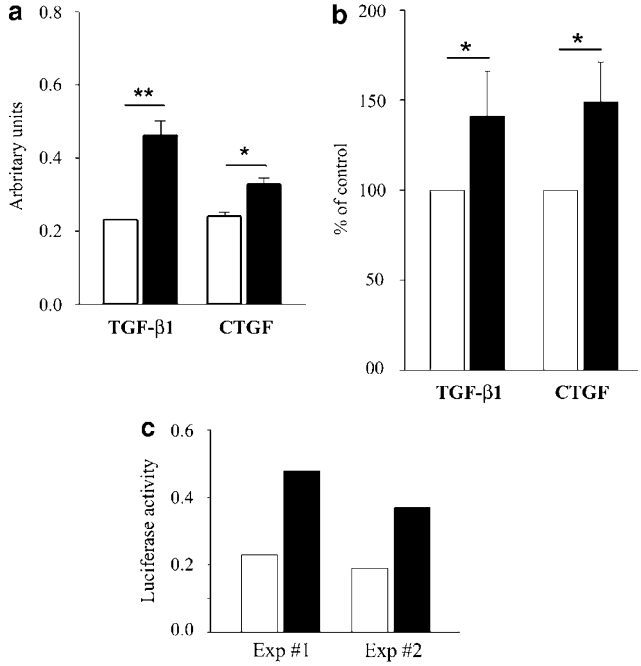

Figure 7 Analysis of TGF-β1 and CTGF during metanephroi development. *In vivo* expression pattern of TGF-β1 and CTGF was assessed by quantitative RT-PCR in 16 dpc metanephroi obtained from control rats (**a**, open bars) and from diabetic mothers (black bars). *In vitro*, the expression of TGF-β1 and CTGF (**b**) in metanephric explants cultivated with 7 mM (open bars) or 28 mM (black bars) glucose were assessed by quantitative RT-PCR. Histograms represent quantification of mRNA expressed as percentage of control values in paired metanephroi (mean $\pm$ s.e.m. from four independent experiments). $^{*}P<0.05$ as compared to control value. (**c**) Conditioned media from the metanephroi cultivated in high glucose condition upregulate TGF-β activity in ATDC5 cells transfected with the TGF-β responsive promoter construct (CAGA)9-MLP-Luc (results from two independent experiments). $^{**}P<0.01$.

Our major result is that type IV collagenase system is decreased in altered diabetic nephrogenesis. Transcription levels and activities of MMP-2 and MMP-9 are strongly reduced in fetal metanephric kidneys issued from STZ-induced diabetic rats. Furthermore, increasing glucose concentration in rat metanephric organ culture media results in a dramatic reduction of MMP-2 and MMP-9 expression within the explant together with an impairment of both explant growth and UB branching. Taken together, these two sets of experiments strongly suggest that impaired type IV collagenase system is involved in diabetic altered nephrogenesis, through abnormal ECM turnover leading to decreased UB branching.

In adult diabetic nephropathy, alteration of type IV collagenase system has been clearly observed.[42] A regulation of collagenase type IV system by TGF-β/CTGF system has

recently been of glomerulosclerosis in diabetes.[23,27] Furthermore *in vitro* studies have demonstrated that high glucose concentration can alter the expression of some MMPs and increase the expression of TIMPs in renal cells.[21,23,24] In fetal kidney, TGF-β1 mRNA has been localized in the UB and in the differentiating mesenchyme and is crucial for the branching of the UB.[40,43–47] In the present study, we clearly demonstrate for the first time that hyperglycemia increases both *in vivo* and *in vitro* the expression of TGF-β1 and of CTGF in renal tissue obtained from diabetic fetuses and from metanephroi cultivated with high glucose concentration. In addition, we show that conditioned medium taken from metanephroi cultured in high glucose concentration increases TGF-β responsive promoter activity in cells transfected with reporter construct (CAGA)9-MLP-Luc. This indicates that the enhancement of TGF-β1 mRNA expression results in enhancement of functional TGF-β. These observations are consistent with recent data[25] reporting that CTGF expression is increased by glucose in renal epithelial cells and that this increase is mediated by TGF-β. Altogether these results strongly suggest a role of TGF-β and CTGF in the alteration of kidney development during gestational diabetes. Therefore several mechanisms, either direct or ECM mediated might underly the inhibitory effect of TGF-β and CTGF on nephrogenesis during maternal diabetes.[4,48]

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