Lack of Type VIII Collagen in Mice Ameliorates Diabetic Nephropathy

Short title: α1 (VIII) Collagen in Diabetic Nephropathy

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**Objective** - Key features of diabetic nephropathy (DN) include the accumulation of extracellular matrix proteins. Recent studies noted an increased expression of type VIII collagen in glomeruli and the tubulointerstitium of diabetic kidneys. The objectives of this study were to assess whether type VIII collagen affects the development of DN and to determine type VIII collagen dependent pathways in DN in the mouse model of streptozotocin induced diabetes.

**Research Design and Methods** - Diabetes was induced by streptozotocin injections in collagen VIII deficient or wildtype mice. Functional and histological analyses were performed 40 days after induction of diabetes. Type VIII collagen expression was assessed by Northern blots, immunohistochemistry and real-time PCR. Proliferation of primary mesangial cells was measured by thymidine incorporation and direct cell counting. Expression of phosphorylated Erk 1, 2 kinase and p27Kip1 was assessed by Western blots. Finally, Col8a1 was stably overexpressed in mesangial cells.

**Results** - Diabetic wildtype mice showed a strong renal induction of type VIII collagen. Diabetic Col8a1−/Col8a2− animals revealed a reduced mesangial expansion, cellularity and extracellular matrix expansion compared to the wildtype. This was associated with less albuminuria. High glucose medium as well as various cytokines induced Col8a1 in cultured mesangial cells. Col8a1−/Col8a2− mesangial cells revealed a decreased proliferation, less phosphorylation of Erk 1,2 and increased p27Kip1 expression. Overexpression of Col8a1 in mesangial cells induced proliferation.

**Conclusions** - Lack of type VIII collagen confers renoprotection in DN. One mechanism might be that type VIII collagen permits and / or fosters mesangial cell proliferation in early DN.
Diabetic nephropathy is the most common cause of end-stage renal failure leading to dialysis. Glomerular lesions are characterized by expansion of the mesangial matrix and thickening of peripheral glomerular basement membranes due to the synthesis and accumulation of extracellular matrix (ECM) (1; 2). The degree of mesangial matrix expansion correlates with the progressive decline in the glomerular capillary surface area available for filtration and hence with the glomerular filtration rate (3). Early changes include a confined proliferation of mesangial cells followed by cell cycle arrest and hypertrophy (3-8). Several growth factors have been implicated in this process, among them transforming growth factor beta1 (TGF-β1) and platelet-derived growth factor (PDGF-BB) (4; 9; 10). During early stages, PDGF-BB potently increases proliferation and matrix synthesis of mesangial cells and induces the expression of TGF-β1 (4; 5; 11).

Upregulation of the PDGF-BB pathway has been shown in kidneys from patients with diabetic nephropathy as well as in experimental models of diabetic nephropathy (12; 13). Further, PDGF-receptor antagonists attenuate diabetic nephropathy (4). Activation of the TGF-β1 loop leads to cell cycle arrest, induction of cyclin-dependent kinase inhibitors and further ECM synthesis (3; 14).

Type VIII collagen, a non-fibrillar short-chain collagen, is a structural component of many extracellular matrices (15-17). Two highly homologous polypeptides, α1(VIII) and α2(VIII), form either homotrimeric or heterotrimeric molecules (18-20). It is involved in cross-talks between cells and the surrounding matrix by modulating diverse cellular responses such as proliferation, adhesion, migration, chemotaxis and metalloproteinase synthesis (21-23). Especially, it is highly expressed by vascular smooth muscle cells in response to PDGF-BB and is thought to be a key component of vascular remodeling (24-27). In healthy kidneys expression of type VIII collagen has been demonstrated in glomerular arterioles, larger branches of renal arteries as well as in rat glomeruli and mesangial cell in vitro (28; 29). An increased mRNA as well as protein expression has been noted in glomeruli and the tubulointerstitium of kidney biopsies with diabetic nephropathy (30; 31). The functional role of collagen VIII, especially in the early phase of the disease has not been investigated and remains obscure.

To address the role of type VIII collagen in the pathogenesis of diabetic nephropathy we applied the streptozotocin (STZ) model to mice with homozygous deletions of both collagen VIII genes and compared them with wildtype mice. The objectives of this study were to assess whether collagen VIII-dependent pathways are involved in the development of diabetic nephropathy and in various cellular and molecular processes associated with this disorder.

RESEARCH DESIGN AND METHODS

Animals. Animal experiments were approved by the local Animal Care Committee of the University of Hamburg and done in accordance with the German Animal Protection Law. Col8a1−/−/Col8a2−/− mice crossed for at least 20 generations into the C57Bl/6 background (21) and wildtype mice were maintained in a pathogen-free facility. All animals had free access to water and were on the standard rodent chow. Systolic blood pressure was measured using tail cuff plethysmography (TSE Systems, Bad Homburg, Germany).

Disease Model. Eight to ten-week-old male mice were randomly divided into groups treated with streptozotocin (STZ, Sigma, Deisenhofen, Germany) or left untreated. STZ was dissolved in sterile citrate buffer and injected intraperitoneally (150 mg/kg body weight) within 10 minutes of preparation on
three consecutive days. To render animals hyperglycemic without becoming ketoacidotic, a subcutaneous insulin implant (Linshin, Ontario, Canada) was administered. 15 Col8a1−/Col8a2− mice and 12 wildtype mice were treated with STZ, 7 Col8a1−/Col8a2− mice and 10 wildtype mice were left untreated. Urinary glucose levels (Diabur Test 5000, Roche, Mannheim, Germany) and body weight were examined at the beginning and the end of this study. Venous blood glucose concentrations were measured with a B-glucose analyzer (HemoCue, Ängelholm, Sweden). Urine samples were collected in metabolic cages at baseline and prior to sacrifice. Mice were sacrificed after 40 days. BUN was measured by a multianalyzer (Hitachi, Ramsey, USA).

\textbf{Quantification of albuminuria.} 2 µl of urine was placed in 18 µl of Laemmli buffer, boiled and loaded on a 12% SDS-PAGE gel. Gels were stained by Coomassie blue following standard procedures and pictures were taken of native gels. The albumin band with a molecular weight of 66.2kDa was assessed densitometrically as described (32).

\textbf{Histology, immunohistochemistry, and immunofluorescence.} Kidneys were fixed in 10% buffered formalin. 4 µm sections were stained with hematoxylin / eosin and periodic-acid-Schiff reagent (PAS). For immunohistochemistry paraffin sections were incubated with protease XXIV (15 minutes, 5 mg/ml), blocked with 5% normal horse or goat serum (30 minutes, room temperature), incubated with anti-laminin (1:3600, overnight, 4°C) or anti-collagen IV (both from Southern Biotechnology, Eching, Germany, 1:600, overnight, 4°C), followed by biotinylated donkey IgG (1:400, 30 minutes, room temperature). Signal amplification was performed with ABC-AP (Vector Laboratory, Loerrach, Germany) according to the manufacturer's instructions using Neufuchsin as a substrate. For immunofluorescence, kidneys were fixed in 10% buffered formalin, infiltrated with 20% sucrose and frozen in OCT. 5µm cryosections were rehydrated, treated with proteinase XXIV, blocked in 5% goat serum in PBS and incubated with rabbit anti-EGFP antibodies (Molecular probes, Karlsruhe, Germany, 1:500, 1 hour, 37°C). A nonimmune rabbit serum was used as a control. Fluorescein-anti-rabbit IgG was applied as secondary antibody. For double immunofluorescence, staining with an anti-EGFP antibody was performed as above; secondary antibody was a Texas-Red-anti-rabbit IgG. Sections were further incubated with anti-CD31/PECAM-1 (BD Biosciences, Franklin Lakes, NJ, USA, 1 hour, room temperature), secondary antibody was a fluorescein-anti-rat antibody.

\textbf{Morphometric analysis.} 20 glomerular cross sections/mouse were photographed with an Axioscope microscope equipped with an AxioCam and evaluated with KS300.1 software (all Zeiss, Oberkochen, Germany) in a double blinded fashion. The area of the glomerular cross section, the glomerular tuft as well as the number of nuclei were measured. Results are expressed as % glomerular tuft area and number of nuclei/glomerular cross section. Capillary density was measured by taking twenty pictures of each section stained against CD31 and evaluation was done with Image J64.

\textbf{Cell culture, transfection and Western blot analysis.} Isolation of RNA and Northern blots. A detailed description can be downloaded online.

\textbf{Isolation and characterization of mouse mesangial cells (MMC).} Two different isolates of wildtype and Col8a1−/Col8a2− MMCs were released from glomeruli using a modification of a published method (33). In brief, for each experiment two wildtype and two knockout mice were sacrificed and immediately perfused with 8 x 10⁷ Dynalbeads® (Dynal, Invitrogen, Karlsruhe, Germany) diluted in 40 ml of HBSS through the heart. Kidneys were removed, minced into
1 mm$^3$ pieces and digested in 1 mg/ml collagenase A (Roche) and 100 U/ml deoxyribonuclease I in HBSS at 37°C for 30 min. The tissue was gently pressed through a 90 µm cell strainer and washed with 5 ml of HBSS. The filtrate was passed through a new cell strainer without pressing and washed again. The cell suspension was then centrifuged at 200 x g for 5 min and resuspended in 2 ml of HBSS. Finally, glomeruli containing Dynalbeads® were gathered by a magnetic particle concentrator and washed three times with HBSS. Collagenase digested glomeruli were seeded into cell culture dishes and maintained in DMEM, 10% serum and 1% glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen) at 37°C and 5% CO$_2$ for 6 days. MMC were passaged every 4 to 5 days.

For characterization, cells were grown on glass coverslips, fixed with 10% formalin and photographed with a phase contrast microscope, or methanol (10 minutes on ice) for immunostainings. Cells were washed with PBS, blocked with 3% BSA in PBS (30 minutes, room temperature), incubated with primary antibodies (1 hour, 37°C), and fluorescein labeled anti-mouse-, anti-rat-, or anti-rabbit IgG (1:200, 1 hour, 37°C). Primary antibodies were anti-collagen VIII (Seikagaku, Tokyo, Japan), anti-EGFP (Molecular Probes), anti-vimentin, anti-desmin, anti-sm actin (Sigma), anti-CD31/PECAM-1, anti-collagen IV (ICN, Northeim, Germany). Isotype matched IgG and nonimmune rabbit IgG were used as controls. Pictures were taken at 200x magnification with an Axioscope fluorescence microscope.

**Thymidine incorporation and growth curves.** MMC (passages 4-10) were plated on 96-well plates at a density of 3x10$^3$ cells/well and maintained in DMEM supplemented with 100 mg/dl glucose, 10% FCS for 24 hours to allow cell attachment. Serum free medium was then added for 24 hours to synchronize the cells in G0 phase. DNA synthesis was measured by [$^3$H]thymidine incorporation: 2 µCi/ml [$^3$H]thymidine was added for 16 h in the presence or absence of either 450 mg/dl glucose or 50 ng/ml PDGF-BB. Cells were washed twice with PBS, trypsinized, and harvested onto a filterpaper using an automated cell harvester (Dynatech Labs., Chantilly, VA). [$^3$H]thymidine incorporation was measured in a scintillation counter. Cell proliferation was further measured using the MTT assay (ROCHE Diagnostics). Results were plotted as means of twelve different values. Experiments were repeated three times with two different cell preparations. In a second set of experiments, MMC (15 x 10$^4$) were seeded into 6 well plates, stimulated as described above, trypsinized and counted using a hemocytometer. D-mannose (450 mg/dl) served as an osmotic control.

**Quantitative real-time PCR.** Rested MMCs were either stimulated with 25 ng/ml epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), transforming growth factor (TGF-β1, all from PeproTech, Hamburg, Germany) or PDGF-BB for 12, 24 and 48 hours or treated as described for northern blots. RNA was purified using a RNA extraction KIT. For quantitative PCR amplifications of Col8a1, DNase I treated first-strand cDNA was used with components of the SYBR Green JumpStart Taq Ready Mix (Sigma) on an AbiPrism NN8650. The cycling parameters were 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. In each experiment, three or more identical PCR reactions of Col8a1 and the control RNA 18S were run using the primers for 18S:sense: 5' -CACGGCCGGTACAGTGAAAC –3'; antisense: 5' -AGAGGAGCGAGCGACCAAA –3' and for Col8a1: sense: 5' -TCTGCCACCTCAAATCCCTCCTCA –3'; antisense: 5' -TCTCCCGCAAAACTGGCTAACC-3'. The
data were calculated by the comparative Ct method (2-[delta][delta]Ct method) by which [delta][delta]Ct = [delta]Ct sample - [delta]Ct reference. Threshold cycles were determined using the default threshold levels and the average threshold cycles were normalized for amplification of 18S as an internal control to correct for small variations in RNA quantity and cDNA synthesis. Relative expression levels were normalized to the unstimulated control. Melting curves and 3% Nusieve agarose gel electrophoresis were used to verify the absence of nonspecific PCR products. Three separate experiments were performed for each experiment and one representative experiment is shown.

**Statistical Analysis:** All data are presented as the means ± SEM. Statistical significance between multiple groups was tested with the Kruskal-Wallis test. Individual groups were subsequently tested using the Wilcoxon-Mann-Whitney test. A p value of <0.05 was considered significant. Experiments that did not yield enough independent data points for statistical analysis due to the experimental setup were repeated three times; one representative experiment is shown.

**RESULTS**

**STZ diabetes in wildtype and Col8a1''/Col8a2'' mice:** Type VIII collagen was shown to be expressed by mesangial cells. We therefore hypothesized that lack of type VIII collagen may alter early mesangial changes seen in diabetic nephropathy. We induced diabetes by the short high dose protocol of repeated STZ injections. Both wildtype and Col8a1''/Col8a2'' mice injected with STZ developed elevated levels of blood glucose as well as glucosuria. At the end of the study the diabetic wildtype and Col8a1''/Col8a2'' mice had failed to gain weight as compared to the non diabetic controls, there was no significant difference between wildtype and knockout mice (Table 1 and data not shown). STZ treated mice developed albuminuria. However, the albumin excretion of diabetic Col8a1''/Col8a2'' mice was significantly lower as compared to the wildtypes (p<0.05, Table 1). Plasma BUN increased significantly with diabetes (p<0.05) but there was not significant difference between genotypes (Figure 1D). Tail plethysmography revealed no significant difference (p<0.8) in systolic blood pressure of untreated wildtype (100 ± 3.8 mm Hg, n=5) and Col8a1''/Col8a2'' mice (99.2 ± 5.8 mm Hg, n=5).

Reduced mesangial expansion and cellularity in Col8a1''/Col8a2'' mice: Histological analysis revealed the characteristic features of STZ-induced diabetic kidney lesions in the wildtype mice as previously described (38). Morphometric quantification of the glomerular tuft area in proportion to the total area of the glomerular cross section displayed a significant increase in diabetic wildtype mice (68.4 ± 4.7%) as compared to non-diabetic controls (60.5 ± 2.0%, p<0.05, Figure 1A). In contrast, diabetic Col8a1''/Col8a2'' mice (62.5 ± 4.3%) had no significant increase compared to nondiabetic Col8a1''/Col8a2'' animals (58.9 ± 1.5%). This finding was supported by an increased immunohistochemical staining for type IV collagen (Figure 2A-D) and laminin (Figure 2E-H).

Additionally diabetic wildtype mice showed significant glomerular hypercellularity (14.6 ± 1.4 nuclei per 1000 glomerular µm² vs. 10.3 ± 1.2 in non-diabetic wildtype mice, p<0.05) while there was no difference in STZ Col8a1''/Col8a2'' mice (12 ± 1.6 vs. 10.7 ± 1.7, Figure 1B). Examples of PAS-stainings are shown in Figure 1E-H. No significant difference was observed in the glomerular volume (Figure 1C). The interstitial capillary density showed no significant difference (CD31 pos. pixels (%): WT CON: 1.37 ± 0.45; WT STZ: 1.37 ± 0.52; KO CON: 1.49 ± 0.51; KO STZ: 1.46 ± 0.45, not significant, n=10-12 for each group).
Expression of type VIII collagen in glomeruli of STZ wildtype mice: Type VIII collagen was detected by immunofluorescence in the mesangium, the interstitium, and around blood vessels in diabetic wildtype mice whereas control mice only stained within the adventitia and the endothelium of arteries (Figure 2I-K). As expected no staining was detectable in Col8a1−/Col8a2− mice (Figure 2L). Since the knockout mice contain an EGFP gene driven by the Col8a1 promotor, we also studied the EGFP expression in STZ and control Col8a1−/Col8a2− mice. The distribution was comparable to the wildtype mice both in the STZ and the control group (Figure 2M-P). EGFP did not colocalize with CD31/PECAM-1 indicating that type VIII collagen is not synthesized by glomerular endothelial cells (Figure 2Q).

High glucose induces Col8a1 but not Col8a2 in mesangial and tubular epithelial cells: To further investigate potential mechanisms we evaluated mRNA expression of Col8a1 and Col8a2 in mesangial (MMC), glomerular endothelial (GER), tubular epithelial cells (MCT) under high glucose conditions. Northern blot analysis revealed an increase of Col8a1 in MMC and MCT after 24 hours whereas Col8a2 expression was not detectable (Figure 3A). A time course experiment in MMC showed that Col8a1 mRNA expression under high glucose conditions was already present at 6 hours (Figure 3B-C). A real time PCR was performed to quantify this effect. Col8a1 mRNA expression was raised after 6 hours, and peaked at 24 hours. Increased D-mannose concentrations to equiosmolar concentrations had no effect indicating that high glucose effect is independent of the medium osmolarity (Figure 3D).

PDGF-BB is an early regulator of Col8a1 in MMC: Since growth factors such as PDGF-BB and TGF-β1 (4; 10) have been implicated in the pathogenesis of diabetic glomerulosclerosis we determined the response of MMC to different cytokines by real-time PCR. Wildtype MMC were stimulated with EGF, bFGF, PDGF-BB or TGF-β1 (Figure 4). At 12 hours PDGF-BB (2.0 ± 0.4; p<0.05) and bFGF (1.8 ± 0.1; p<0.05) induced Col8a1 about two-fold while there was no TGF-β1 (1.0 ± 0.6) or EGF effect (0.7 ± 0.0). At 24 and 48 hours TGF-β1 (24 hours:4.2 ± 0.2; 48 hours:1.8 ± 0.2, p<0.005) was the strongest inductor. PDGF-BB and bFGF had a sustained effect at 24 hours but not at 48 hours. These results suggest that PDGF-BB and bFGF are early regulators of Col8a1 expression while TGF-β1 is more important at a later time point.

Decreased proliferation of Col8a1−/Col8a2− MMC: We isolated Col8a1−/Col8a2− and wildtype MMC to compare their ability to proliferate in response to glucose and PDGF-BB. Characterisation of the primary mesangial cell cultures showed the expected elongated stellate-shape in wildtype and Col8a1+/Col8a2− mice (Figure 5). By immunofluorescence the cells stained positive for vimentin, desmin, sm-actin and were negative for CD31/PECAM-1. Wildtype MMC were positive for type VIII collagen while Col8a1−/Col8a2− MMC stained for EGFP. [3H]Thymidine incorporation and cell counts were used as an index of MMC proliferation (Figure 6A, B). High glucose stimulated cell proliferation significantly less in Col8a1−/Col8a2− MMC than in wildtype MMC. Stimulation with PDGF-BB had an identical effect (Figure 6 C, D). D-Mannose had no impact on cell proliferation (Figure 6I). Transfection of Col8A1 into Col8a1−/Col8a2− MMC reversed the phenotype and prevented the decrease of proliferation in the Col8a1+/Col8a2− cells after challenge with high glucose or PDGF-BB (Figure 6E-H).

The discoidin domain receptor tyrosine kinase (DDR1) functions as a collagen receptor on MMC and signals by MAPK phosphorylation (39). Since Col8a1−...
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/Col8a2− MMC proliferated at a slower pace than wildtype MMC, we evaluated the amount of Erk 1,2 (p42/p44) MAPK phosphorylation (Figure 6J). Compared to wildtype, Col8a1−/Col8a2− cells had a lower pp42/44 level at the resting state. Upon stimulation with PDGF-BB Col8a1−/Col8a2− MMC showed a markedly decreased p42/44 phosphorylation compared to wildtype MMC. Since an increased proliferation is associated with a decrease of the cell cycle inhibitor p27Kip1 and MAPK directly phosphorylates and stabilized p27Kip1 (40), we asked if type VIII collagen also alters the expression of p27Kip1 (Figure 6K). As predicted PDGF-BB induced down-regulation of p27Kip1 in wildtype MMC. In contrast the levels of p27Kip1 were increased in Col8a1−/Col8a2− MMC and stimulation with PDGF-BB had no further effect.

Overexpression of Col8a1 leads to an increased proliferation of MMC: A MMC cell-line overexpressing Col8a1 was generated. A 2.5 (± 0.2) fold mRNA overexpression was determined by real-time PCR (data not shown). Stimulation with high glucose or PDGF-BB for 24 hours resulted in an significant increase in thymidine incorporation and cell counts compared to mock transfected cells (Figure 7A-D). A mild induction of p42/44 was found which was enhanced upon stimulation with PDGF-BB (Figure 7E).

DISCUSSION

Mesangial cells are a major player in the maintenance of glomerular integrity. This is accomplished in part through expression of extracellular matrix proteins and growth factors. In diabetic glomerulosclerosis elevated glucose results in pronounced alterations of mesangial cell function with an early limited proliferation of mesangial cells (4-8), followed by cell cycle arrest, hypertrophy (3), excess production and decreased degradation of extracellular matrix (ECM) components including collagens, fibronectin, laminin and proteoglycans (41). These multiple steps are orchestrated by growth factors and hormones (10; 12).

Several features of type VIII collagen suggest that it could play an important role in mesangial cell function and the pathogenesis of diabetic glomerulosclerosis. It is part of the mesangial matrix in diabetic nephropathy (30; 31), mesangial cells synthesize α1(VIII) collagen (16; 29), and type VIII collagen has been shown to modulate cell proliferation of diverse cells including vascular smooth muscle cells (23; 27; 42). We used genetically modified mice deficient in both Col8 genes to test its role in diabetic nephropathy in the STZ model (21). Several principal finding emerge from our study: (1) in the absence of type VIII collagen the severity of the glomerular changes is markedly attenuated and albuminuria is improved, (2) high glucose, PDGF-BB and TGF-β1 are strong inducers of Col8a1 gene expression in MMC, and (3) α1(VIII) collagen permits and/or stimulates MMC proliferation.

Our in vivo data clearly show that type VIII collagen is necessary for mesangial matrix expansion as well as for hypercellularity. Type VIII collagen accumulated in the mesangium of diabetic wildtype mice. This is consistent with previous reports of human kidney biopsies (30; 31). Our in vitro data help to explain this difference. High glucose increases Col8a1 mRNA expression by mesangial cells while Col8a2 mRNA was not detectable. This suggests the formation of α1(VIII) collagen homotrimers in the diabetic mesangium. Growth factors such as PDGF-BB, bFGF, and TGF-β1 have been implicated in the pathogenesis of diabetic glomerulosclerosis (4; 9), and the expression of Col8a1 in vascular smooth muscle cells has been described after stimulation with these factors (25; 26). Therefore it is not surprising that PDGF-BB and bFGF act as early regulators of
Col8a1 expression in MMC followed by TGF-β1. This kinetic parallels the early activation of a PDGF loop that, in turn, causes an increase of TGF-β1 expression, thus modulating both mesangial cell proliferation and matrix production (11; 43). Induction of diabetic nephropathy is associated with an increased proliferation of mesangial cells and treatment of diabetic mice with antagonists for the PDGF receptor reduced the number of proliferating cells (4). MMC with inactivated Col8a1 and Col8a2 genes proliferate less in response to high glucose or growth factors than wildtype MMC. In addition, overexpression of α1(VIII) collagen results in an increased cell growth. Type VIII collagen is expressed by a number of rapidly proliferating cells and it promotes vascular smooth muscle cell migration and proliferation during vascular remodelling (21-24; 27). Therefore pericellular type VIII collagen may help maintain the mesangial cells in a proliferative state during early development of diabetic nephropathy. This is consistent with the finding that Col8a1−/Col8a2− vascular smooth muscle cells exhibited lower proliferation rates than wildtype cells when plated on type I collagen (27). This suggests that endogenously produced type VIII collagen allows smooth muscle cells to overcome the inhibitory effects of type I collagen on proliferation, which may also be important in the context of diabetic nephropathy.

Since systolic blood pressure was not significantly different in wildtype and Col8a1−/Col8a2− mice and no significant change in interstitial capillary density was observed, we think that the change in mesangium phenotype is the major mechanism explaining the attenuation of diabetic nephropathy in Col8a1−/Col8a2− mice.

Over the last years toxicity has become a major concern in the STZ model of diabetes mellitus (38). STZ has a dose-dependent tubular toxicity resulting in renal dysfunction and acute tubular necrosis (45). Therefore studies of renal function and tubulointerstitial changes should be interpreted with much caution because it is difficult to dissect the contribution of hyperglycemia and toxicity especially in high dose protocols as used in our study. However, to our knowledge, there is no evidence, that the diffuse glomerulosclerosis in the STZ model can also be attributed to a toxic side effect of STZ. In humans STZ does not cause diabetes mellitus (46) and is used to treat metastatic islet cell carcinomas of the pancreas. Although its renal tubulotoxicity is the major dose limiting side effect of this drug (47), there have been no report of diffuse or nodular glomerulosclerosis in more than 25 years in clinical use. Several mice studies comparing low and high dose protocols showed a similar degree of diffuse glomerulosclerosis in both groups (39; 48). Additionally, blood glucose control by insulin treatment of rats rendered diabetic with STZ prevented diffuse glomerulosclerosis (49).

Mitogen-activated protein kinases (MAPK), including the extracellular signal-regulated protein kinase-1,2 (ERK 1,2; p42/p44), play a key role in the intracellular signal transduction cascade to integrate the transcription of genes responsible for a variety of cellular responses relevant to diabetic nephropathy (44). Our data support a role of type VIII collagen in activating ERK 1,2. This may be achieved through the collagen-receptor discoidin domain receptor 1 (DDR1) that is a key regulator of mesangial cell proliferation and that is stimulated by type VIII collagen (39; 50). While the unstimulated receptor suppresses Erk 1,2 activation, the activated DDR1 induces Erk 1,2 phosphorylation and subsequent proliferation. Mesangial cell proliferation is governed at the level of the cell cycle by regulatory proteins. Specifically, cyclin dependent kinase (CDK) inhibitors including p27Kip1 limit cell proliferation by binding to
and inhibiting cyclin-CDK complexes (37). Consistent with a decreased proliferation in Col8a1−/Col8a2 − MMC the levels of p27Kip1 were increased compared to wildtype mice.

In summary, we provide evidence that type VIII collagen acts as important messenger molecule regulating mesangial cell responses during diabetic nephropathy. The lack of type VIII collagen confers renoprotection in the STZ model of diabetic nephropathy. We conclude that type VIII collagen may function to permit and / or foster mesangial cell proliferation in the early stage of diabetic nephropathy.

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TABLES

Table 1 Variables measured during the study period of STZ-diabetes

| Variable                        | Body weight (g) initial | Body weight (g) end | Blood glucose (mg/dL) | Urinary glucose (g/dL) | Albuminuria (OD / mm²) |
|--------------------------------|-------------------------|---------------------|-----------------------|------------------------|------------------------|
| Col8a1^- / Col8a2^- controls   | 24.6 ± 1.3              | 31.4 ± 1.8          | 128.5 ± 28.7          | 0                      | 0.2 ± 0                |
| Col8a1^- / Col8a2^- STZ        | 27.2 ± 3.5              | 26.9 ± 3.3          | 292.4 ± 66.6          | 3.7 ± 0.9              | 0.3 ± 0.2              |
| Col8a1^+ / Col8a2^+ controls   | 26.9 ± 2.3              | 29.7 ± 2.5          | 155.7 ± 36            | 0                      | 0.1 ± 0                |
| Col8a1^+ / Col8a2^+ STZ        | 26.8 ± 3.4              | 26.2 ± 3.2          | 319 ± 61.2            | 3.8 ± 1                | 0.5 ± 0.3              |

*: P < 0.01 versus Col8^-^- controls, §: P<0.01 versus Col8^-^- controls, #: P<0.05 versus STZ Col8^-^-  

FIGURE LEGENDS

Figure 1 Morphometry of diabetic (black bar) and nondiabetic (blank bar) wildtype and Col8a1^- /Col8a2^- mice. (A) Mesangial tuft area per glomerulus (µm²). (B) Mesangial nuclei per 1000µm². (C) glomerular volume. (D) Plasma BUN (blood urea nitrogen; mg/100ml). Bars represent SEM; *p < 0.05, n=10-12. Representative PAS stainings of nondiabetic wildtype (E) and Col8a1^- /Col8a2^- mice (G) and diabetic wildtype (F) and Col8a1^- /Col8a2^- mice (H). NS: not significant

Figure 2 Light microscopic features of the glomerular lesions and expression of type VIII collagen. Representative glomerulus of an untreated wildtype (A, E) and a Col8a1^- /Col8a2^- mouse (C, G) stained against type IV collagen (A, C) and laminin (E, G). Representative glomerulus of a wildtype (B, F) and a Col8a1^- /Col8a2^- mouse (D, H) treated with STZ and stained against type IV collagen (B, D) and laminin (F, H). Diabetes was associated with an increase in type IV collagen and laminin protein expression. Lack of collagen VIII reduced the accumulation of type IV collagen (C, D) and laminin (G, H) in the glomeruli.

Immunofluorescence staining against collagen VIII in wildtype mice (I, J, K) or EGFP in Col8a1^- / EGFP "knock-in" mice (M, N, O). Strong staining for collagen VIII (I) and EGFP (M) was seen in healthy mice within the adventitia and the endothelium of arteries, whereas no staining was apparent in tubuli or glomeruli. In diabetic mice strong staining within the tubular interstitium, the glomeruli and around arterioles (anti-type VIII collagen: (J, K); anti-EGFP: (N, O)). No staining against type VIII collagen in Col8a1^- /Col8a2^- mice (L) or EGFP in wildtype mice (P). Double immunofluorescence revealed that EGFP (red) only partly colocalized with CD31/PECAM-1 (green), a marker for vascular endothelial cells (Q). Original magnification: 20x.

Figure 3 High glucose induces Col8a1. Increased D-glucose concentrations (from 100mg/dl to 450mg/dl) raised the Col8a1 mRNA in tubular epithelial (MCT) and mesangial (MMC) cells but not in glomerular endothelial cells (GER) in Northern blots (A). High glucose increased the Col8a1 mRNA expression in MMCs after 6 hours (B). mRNA of murine corneal endothelial cell (mCEC) represent a positive control. (C) Representative real time PCR analyses on glucose treated MMCs. Increased D-mannitose concentrations had no effect on the Col8a1 expression (D).
Figure 4  Real-time PCR of Col8a1 mRNA of MMCs in response to different cytokines. Wildtype MMCs were stimulated with 25ng/ml EGF, bFGF, PDGF-BB or TGF-β1 for 12, 24 and 48h. After 12h (A) strong Col8a1 expression was seen by PDGF-BB and bFGF, none by TGF-β1 and EGF. TGF-β1 was the strongest inductor of Col8a1 after 24h followed by PDGF-BB, EGF, and bFGF (B). Stimulation for 48h with TGF-β1 had still an effect on the Col8a1 expression, but none by bFGF, EGF and PDGF-BB (C). Bars represent SEM; *P < 0.05.

Figure 5  Characterization of MMCs. The isolated mesangial cells showed the expected elongated stellate-shape (A, B). An antibody against collagen VIII stained wildtype - (C) but not Col8a1−/Col8a2− MMCs (D) whereas EGFP was stained in Col8a1−/Col8a2− (F) but not wildtype -MMCs (E). Intermediate filaments typical for MMCs stained positive: vimentin (G, H) and desmin (K, L). An antibody against cytoskeletal α smooth muscle actin distinguished MMCs from fibroblasts (I, J). Staining MMCs with antibodies against markers specific for vascular endothelial cells such as CD31/PECAM-1 was negative (M, N). An isotype matched mouse IgG was used as control (O). wildtype: A, C, E, G, I, K, M, O; Col8a1−/Col8a2−: B, D, F, H, J, L, N. Original magnification: 20x

Figure 6  Decreased proliferation of MMCs in Col8a1−/Col8a2− MMCs. Thymidine incorporation by Col8a1−/Col8a2− MMCs was significantly attenuated compared to wildtype controls (after glucose (A) and PDGF-BB stimulation(C)). Cell proliferation was also assessed by cell counts in the presence or absence of high glucose (B) or PDGF-BB (D). Transfection of Col8A1 reversed the phenotype (E-H). Increased mannose concentrations had no impact upon proliferation (I). Further, compared with the wildtype, Col8a1−/Col8a2− cells had lower pp42/pp44 level at the resting state and after stimulation with PDGF-BB in western blots (J). p27kip1 levels were increased in Col8a1−/Col8a2− MMCs and stimulation with PDGF-BB had no further effect (K). *P< 0.01 versus wildtype MMC. Bars represent SEM. NS: not significant

Figure 7  Overexpression of Col8a1 increased proliferation of MMCs. Stimulation with glucose (A) or 50ng/ml PDGF-BB (C) for 24h leads to an increase in thymidine incorporation of Col8a1-MMcs compared to mock transfected MMCs. Cell counts confirmed an increased growth of Col8a1-MMcs compared to mock transfected cells (stimulation with glucose (B), or PDGF-BB (D)). Bars represent SEM. *P< 0.05, **P<0.01. Western blots stained with the phosphorylation specific p42/p44 antibody revealed an induction of p42/p44 in Col8a1-MMcs, which was low in mock transfected MMCs (E).
Figure 1

(A) Glomerular tuft (% glomerularea)

(B) Nuclei / 1000 \( \text{\mu}m^2 \)

(C) Glomerular volume (x10 \( \mu\text{m}^3 \))

(D) Plasma BUN (mg/dL)

(E) WT CON

(F) WT STZ

(G) KO CON

(H) KO STZ
Figure 3

A

Co/8a1

Co/8a2

18S

B

Co/8a1

18S

C

D
Figure 4

(A) 12h

(B) 24h

(C) 48h

Where: Control, EGF, FGF, PDGF, TGF

**Relative expression of Col8A1 (% of ND)**
Figure 5
Figure 6

(A) Fold increase (Thymidine incorp.)

(B) Fold increase (number of MMCs)

(C) Fold increase (MTT assay)

(D) Fold increase (number of MMCs)

(E) Fold increase (MTT assay)

(F) Fold increase (number of MMCs)

(G) Fold increase (MTT assay)

(H) Fold increase (number of MMCs)

(I) Fold increase (MTT assay)

(J) pp42/44

(K) p27Kip1

β-actin
Figure 7

A, B, C, D: Bar graphs showing fold increase (Thymidine incorp.) and fold increase (number of MMCs) for different conditions.

E: Western blot images for Anti pp42/pp44 and Anti p42/p44 for Col8 MMC and Mock MMC.