The Role of CTGF in Diabetic Nephropathy

Marker, pathogenic factor and target for therapeutic intervention

Peggy Roestenberg

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De rol van CTGF in diabetische nefropathie

Marker, pathogene factor en target voor therapie

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

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	Dr. F.A. van Nieuwenhoven

CONTENTS

LIST OF ABBREVIATIONS

AGE	Advanced glycation end product
AHT	Antihypertensive treatment
BBM	Basement membrane of both pericyte and endothelial cell
BM	Basement membrane
BMI	Body mass index
BMP	Bone morphogenetic protein
BMPR	BMP receptor
CCN	Cystein rich-61/CTGF/Nov (family of genes)
CCN-2	CTGF
CTGF	Connective tissue growth factor
CTGF-N	N-terminal domain of connective tissue growth factor
DM	Diabetes mellitus
DN	Diabetic nephropathy
DR	Diabetic retinopathy
EBM	Endothelial basement membrane
ECM	Extracellular matrix
EGF	Epidermal growth factor
GBM	Glomerular basement membrane
GFR	Glomerular filtration rate
IBM	“Inner” basement membrane
IGF	Insulin-like growth factor
HSPG	Heparan sulphate proteoglycan
LRP	Low-density lipoprotein receptor-related protein
MA	Microalbuminuria
MDRD	Modification of diet in renal disease
MMP	Matrix metalloproteinase
NA	Normoalbuminuria
PAI-1	Plasminogen activator inhibitor 1
PDGF	Platelet-derived growth factor
PKC	Protein kinase C
OBM	“Outer” basement membrane
ODN	Oligodeoxynucleotide
PBM	Pericyte basement membrane
ROS	Reactive oxygen species
sBP	Systolic blood pressure
SMA	Smooth muscle actin
STZ	Streptozotocin

TβR	TGF-β receptor
TGF-β	Transforming growth factor β
TrkA	Tyrosine kinase receptor A
TBP	Tata box-binding protein
TIEG	TGF-β inducible early gene
TIMP	Tissue inhibitor of metalloproteinases
TSP	Thrombospondin
UAE	Urinary albumin excretion
VEGF	Vascular endothelial growth factor
vWF	Von Willebrand factor
VWC	Von Willebrand factor type C domain

Chapter 1

General introduction and outline of the thesis

DIABETES MELLITUS

The number of patients with diabetes mellitus (DM) is rapidly increasing during the last decades. Nowadays around 200 million people are suffering from diabetes worldwide, and the World Health Organization estimates that by the year 2025, the number of diabetic patients will be increased to above 300 million (i.e. 6% of the total population) [1]. Diabetes mellitus is a metabolic disease, which is characterized by high glucose levels in blood (hyperglycemia) and urine (glucosuria). The majority of patients with diabetes mellitus can be classified into two major types: type 1 diabetes mellitus (approximately 10% of all diabetic patients), which is caused by a lack of insulin production due to destruction of the insulin producing β-cells in the pancreas, and type 2 diabetes mellitus (about 90% of all diabetic patients) in which the response to insulin is affected (insulin resistance). Since insulin is an important metabolic hormone, the absence of insulin production itself, or the inadequate response to insulin, severely affects the metabolism of carbohydrates, fat and proteins. Hyperglycemia and glucosuria are hallmarks of both types of diabetes, but also plasma fatty acid levels are increased. A longstanding diabetic state can result in several severe chronic complications. 50% to 80% of the diabetic patients die from cardiovascular complications [1]. Other major complications of both type 1 and type 2 diabetes mellitus are diabetic retinopathy, diabetic neuropathy and diabetic nephropathy. Of interest, cardiovascular complications are particularly prominent in patients with diabetic nephropathy [2].

PATHOGENESIS OF DIABETIC NEPHROPATHY

Diabetic nephropathy (DN) is an important cause of morbidity and mortality in diabetic patients. About 25% to 40% of the patients with DM will eventually develop diabetic nephropathy, mostly within 15-20 years after diagnosis [3, 4].

Diabetic nephropathy is a chronic kidney disease. The basic functional unit of the kidney is the nephron, of which there are more than a million within the cortex and medulla of each normal adult human kidney (figure 1A and B). Nephrons regulate water and soluble matter in the body by first filtering the blood under pressure, and then reabsorbing some necessary fluid and molecules back into the blood while secreting other, unneeded molecules. The nephron consists of a glomerulus in which the fluid is filtered and a tubule in which the filtered fluid is converted into urine. The glomerulus consists of a network of up to 50 parallel branching capillaries enclosed in Bowman's capsule (figure 1C). The capillaries are enclosed by podocytes, cells that play a major role in the production of primary urine and are largely responsible for the production of components of the glomerular basement membrane (GBM). In addition, mesangial cells surround the capillaries and provide structural, contractile and synthetic properties regulating the function of the glomerulus.

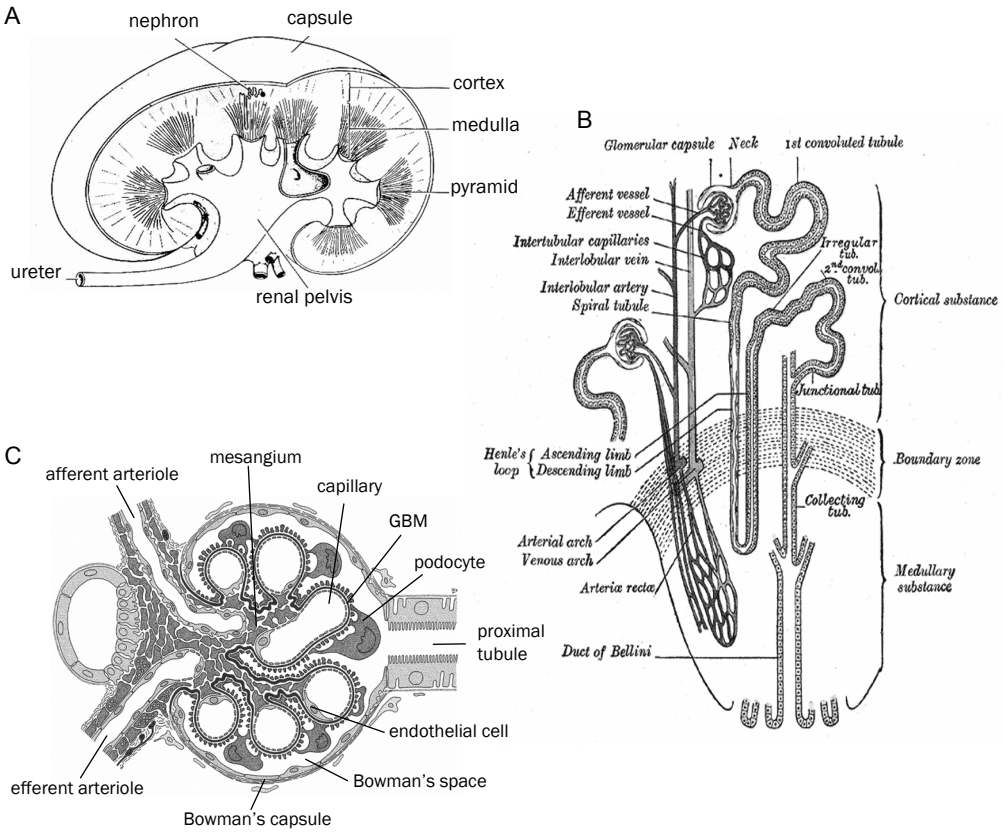

Figure 1: Schematic overview of the anatomy of the kidney. A: Kidney, B: Nephron (adapted from Gray [46]) C: Glomerulus (adapted from Kriz [47]).

Early characteristics of DN are increased urine production (hyperfiltration), microalbuminuria (albumin excretion 30-300 mg/day), renal and glomerular hypertrophy, mesangial matrix accumulation and thickening of the glomerular basement membrane [5]. In later stages, when DN progresses, patients develop proteinuria (albumin excretion > 300 mg/day), and their glomerular filtration rate (GFR) declines, eventually leading to end-stage renal disease. Hyperglycemia, hyperlipidemia, hypertension, and also proteinuria itself, contribute to progression of renal damage. The genetic background of the patients is an important factor since the incidence of this complication is much higher in some specific ethnic groups [6]. Moreover, several genes and loci have been associated with susceptibility for development of DN [7].

The current understanding of the pathophysiology of DN suggests that complex interactions exist between multiple pathways. These include abnormalities in glucose transport mechanisms, increased activity of specific intracellular metabolic pathways, activation of protein kinase C isoforms, formation of reactive oxygen species (ROS),

increased production of advanced glycation end products (AGEs) and, altered activity of a variety of growth factors and cytokines [8]. Functional candidate gene polymorphisms that alter the level of activity within these pathways have been studied, however, to date, little consistent evidence has been found for their contribution to the risk for DN [9].

ROLE OF GROWTH FACTORS IN DIABETIC NEPHROPATHY

Several different growth factors are known to be involved in the development of diabetic complications [10-13]. Disturbed growth factor signaling adversely affects tissue function and influences the extracellular matrix (ECM). Changes in the amount and composition of the ECM are observed in all complications of diabetes and have been attributed a central role in their progression.

One of the major growth factors involved in ECM accumulation in fibrotic disorders, including DN, is transforming growth factor-β (TGF-β) [10]. TGF-β expression is especially increased in mesangial cells of diabetic glomeruli [14, 15], but increased TGF-β expression in glomerular endothelial cells has also been reported [16]. Inhibition of TGF-β results in prevention of fibrosis under experimental diabetic conditions. Treatment of diabetic mice with neutralizing antibodies against TGF-β attenuated both the increased renal TGF-β expression and the increased TGF-β protein levels, and reduced the diabetes-associated renal and glomerular hypertrophy as well as the increased renal expression of collagen IV and fibronectin [17]. TGF-β neutralizing antibodies were able to attenuate mesangial matrix expansion and GBM thickening, and even partially reversed established DN in db/db mice [18, 19]. However, TGF-β has also critical anti-proliferative (tumor-suppressor) and anti-inflammatory effects. For example, TGF-β1 knockout mice die at an age of 3-4 weeks due to a multifocal inflammatory disease in almost all tissues [20], and defective TGF-β signaling is involved in development of several (epithelial) tumors. This makes TGF-β a less suitable target for therapeutic intervention.

Other growth factors that have been linked to fibrotic processes include connective tissue growth factor (CTGF), insulin-like growth factors (IGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) [12, 21, 22]. Expression of all these growth factors was shown to be increased in the diabetic kidney although there are remarkable differences in their expression patterns. Increased PDGF-BB expression in diabetic glomeruli seems mainly localized to the mesangial cells [23], while VEGF overexpression is present in podocytes [15, 24], and EGF expression is localized in renal tubules [25]. Increased expression of IGF-I has been reported in glomerular and tubular cells [26]. As for CTGF expression, this has been reported mainly in the glomeruli of diabetic patients and experimental diabetic animals [27-29], but also prominent tubular expression has been noted [30].

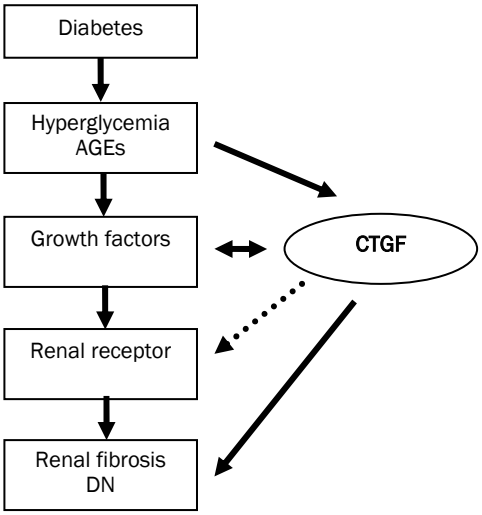

Figure 2: Schematic overview of the involvement of CTGF in the pathogenesis of DN. Hyperglycemia and advanced glycation end products (AGEs) induce the expression of growth factors including CTGF. Furthermore, CTGF expression is induced by other diabetes-induced growth factors. Interaction of CTGF with other growth factors as well as direct binding of CTGF to renal cell surface receptor proteins results in extracellular matrix accumulation in the kidney (renal fibrosis) and eventually DN.

The presence of signaling receptors for all growth factors mentioned above has been reported in the kidney [31-37]. CTGF occupies a special position in this field since most of its action seems to be indirect, i.e. through modulation of signaling activity of other growth factors, including TGF-β, BMP(-4), VEGF, and IGF-1 [38-40] (Fig 2). The importance of CTGF in fibrosis is attributed principally to its role as an important downstream mediator of pro-fibrotic TGF-β action.

Besides interactions with growth factors, CTGF can also act through interaction with cell surface integrins [41-43]. Recently, also direct CTGF-induced signaling through the low-density lipoprotein receptor related protein (LRP), as well as through the tyrosine kinase A (TrkA)/nerve growth factor (NGF)-receptor, has been described [44, 45]. However, the contribution of such signaling to fibrosis and in particular DN is still largely unknown.

AIM AND OUTLINE OF THIS THESIS

The aim of the studies described in this thesis was to investigate the role of CTGF in the development and progression of diabetic nephropathy. Hereto, we focused in particular on the possible function of CTGF as a marker, pathogenic factor, and target for therapeutic intervention of DN.

In this chapter (**chapter 1**) a brief introduction is given about DN and the role of growth factors in this disease. The current knowledge about the role of CTGF in DN is reviewed in more detail in **chapter 2**, as well as the perspectives of CTGF as marker and therapeutic target of DN.

In **chapters 3 and 4** the perspectives of plasma CTGF level as marker for DN are studied. In **chapter 3** the relation of CTGF with general patient characteristics and markers for nephropathy is probed in a relatively small pilot study of diabetic patients with different grades of DN. **Chapter 4** describes a large study of plasma CTGF levels in diabetic patients with and without DN.

In **chapter 5** we study the temporal expression profile and distribution pattern of CTGF during development of DN following streptozotocin (STZ)-induced diabetes in mice. Therefore, we determined CTGF levels in plasma and urine as well as CTGF mRNA and protein levels in renal tissue and other organs. **Chapter 6** describes a study of the pathogenic role of CTGF in DN, and of its importance as a target for therapeutic intervention. For this, we examined the effects of reduced CTGF expression caused by disruption of one of the CTGF alleles on the development of DN in STZ-treated mice. In **chapter 7,** a similar study is presented, now addressing the effect of lower CTGF expression on development of diabetic retinopathy.

In **chapter 8** we present an overview of the possible mechanisms by which CTGF might participate in diabetes-induced thickening of the renal glomerular basement membrane (GBM).

Finally, in **chapter 9** the data presented in this thesis are briefly summarized and discussed.

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Chapter 2

The role of connective tissue growth factor in diabetic nephropathy

Peggy Roestenberg, Frans A. van Nieuwenhoven and Roel Goldschmeding

Manuscript in preparation

CTGF (CCN-2) is a 36-38 kD secreted protein belonging to the Cyr61/CTGF/NOV (CCN) family of matricellular proteins [1]. The exact size of human CTGF appears to be based on N-linked glycosylation of the protein in humans [2]. Since glycosylation is absent in all other species, it appears that the conserved functions of CTGF are independent of inter-species differences in carbohydrate modifications of the protein [2].
CCN proteins are characterized by an extraordinary high content of cysteine residues (>10%) and an absolute conservation of the position of these 38 cysteine residues in the peptide sequence [3]. Furthermore, they are mosaic proteins made up of four conserved modules derived from other, unrelated extracellular matrix proteins (figure 1) [1]. This modular configuration might not only dictate actions on target cells but also bioavailability, half life, binding to other protein moieties and regulation in time and

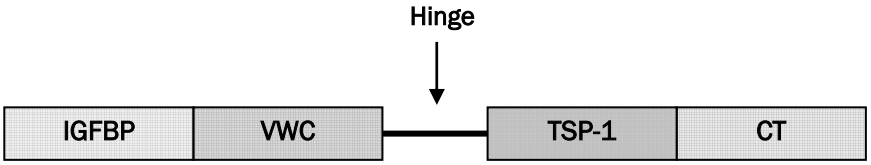

Figure 1: Structure of the CTGF protein. IGFBP: insulin-like growth factor binding protein domain, VWC: von Willebrand factor type C domain, TSP-1: thrombospondin-1 domain, CT: C-terminal domain.

space. It has been shown that the different modules of CTGF can interact with the cell surface, other growth factors, or ECM components (figure 2). CTGF is subject to cleavage by proteases such as elastase, plasmin and several matrix metalloproteinases (MMPs), which adds further to the complexity of its possible regulatory actions [4, 5].
The first module of CTGF is an amino-terminal insulin-like growth factor binding protein domain (IGFBP). This module can interact with IGF-I, although the binding affinity is 2-3 times lower than that of IGF binding proteins 1-6 [6, 7]. The second domain is a cysteine-rich von Willebrand type C (VWC) domain, which has been shown to interact with TGF-β1 and BMP-4. Binding of these growth factors to CTGF enhances TGF-β1 signaling while BMP-4 signaling activity is reduced [8]. The third module is a thrombospondin type 1 repeat (TSP-1). This domain can interact with VEGF, resulting in reduced signaling activity of VEGF [9]. Of interest, cleavage of CTGF by MMPs leads to dissociation of the VEGF-CTGF complex and restoration of the signaling activity of VEGF [10]. In addition, this third module has been implicated in binding of CTGF to the low-density lipoprotein receptor-related protein (LRP) as well as some integrins [11, 12]. The fourth and last module of CTGF is a cysteine-rich carboxy-terminal (CT) domain [3]. This domain has been implicated in binding of CTGF to cell surface and ECM proteins, e.g. integrins and heparan sulphate proteoglycans (HSPGs) [13, 14].

Between module 2 and 3, a cysteine-free hinge region is situated. In this region, CTGF can be cleaved by MMPs and other proteases [5]. Proteolytic cleavage in the hinge region generates 2 fragments with different biological activity [15].

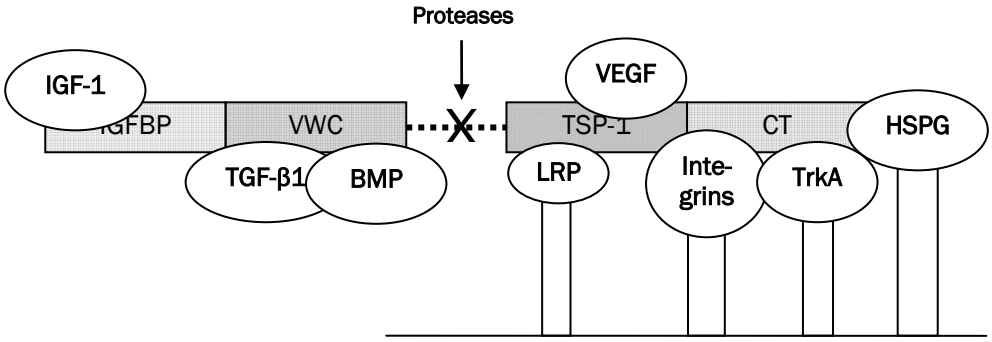

Figure 2: Possible interactions of the CTGF protein with other growth factors and cell surface proteins. IGFBP: insulin-like growth factor binding protein domain, VWC: von Willebrand factor type C domain, TSP-1: thrombospondin-1 domain, CT: C-terminal domain, IGF-I:insulin-like growth factor-I, TGF-β1: transforming growth factor-β1, BMP: bone morphogenetic protein, LRP: low-density lipoprotein receptor-related protein, VEGF: vascular endothelial growth factor, TrkA: tyrosine kinase A, HSPG: heparan sulphate proteoglycans.

The unique biological functions of CTGF and specific fragments thereof may derive in part from the specific interactions with ECM proteins and integrin subtypes expressed by a particular target cell, from the utilization of cell surface heparan sulphate proteoglycans (HSPGs) as co-receptors, and from generation of cross-talk between signaling pathways associated with the different binding partners of individual domains (Figure 2) [1, 15]. Furthermore, specific signaling induced by direct binding and activation of cell surface receptors has been identified for CTGF interaction with several integrins, LRP and TrkA.

LOCALIZATION AND REGULATION OF CTGF EXPRESSION

The localization of CTGF mRNA expression in a variety of organs of adult mice has been visualized by in situ hybridization [16]. This showed that strong and persistent CTGF gene expression was particularly prominent in the mesenchyme of the cardiovascular system (aorta, auricular tissue), in renal glomeruli, the mesenchyme surrounding the ovarian follicles or the testicular tubes in the gonadal tissue, and the subcapsular mesenchyme bordering densely innervated parts of whisker hair vibrissae [16]. CTGF hybridization signals were not observed in the mesenchyme of many other organs including gut, muscle, liver or most parts of the lymphatic tissue. As for epithelial CTGF expression, strong expression was present in the primary (early) ovarian follicles, the

epithelium of the deep uterine glands and on myenteric ganglia neurons. These data suggest a selective and continuous mesenchymal function in the gonads and those tissues attracting very strong vascular supply or peripheral innervation [16].
In healthy adults, CTGF expression is detectable in most kinds of tissues, including heart, brain, placenta, lung, liver, muscle, kidney, and pancreas. Of these tissues, the kidney showed the highest CTGF expression. For example, renal CTGF expression was 30-fold higher than CTGF expression in the brain [17].
CTGF expression is differentially regulated depending on the cell type and condition [18]. In the promotor sequence of CTGF, several functional response elements have been identified. The so-called TGF-β response element (TβRE, BCE-1) was later shown to be important mainly in regulation of basal expression of CTGF [19, 20]. The CTGF promotor also contains a Smad binding element (SBE), which is involved in TGF-β induced CTGF (over)expression [19]. Recently it was shown that TGF-β-induced Smad-dependent induction of CTGF involves FoxO factors as co-factor for DNA binding [21]. Another element of the CTGF promotor is an Sp-1 binding site which has been shown to be involved in TGF-β independent, promotor activity. Targeting Sp1 markedly reduced CTGF expression in dermal fibroblasts of scleroderma patients [22]. Of note, hyperglycemia was shown to enhance glycation of Sp-1 and to increase Sp-1 activity [23]. Other promoter elements of CTGF include binding sites for NFkB, STAT and AP-1, MZF-1 [18], however the possible role of these elements in CTGF overexpression and other conditions is still unclear.
The possible molecular mechanisms implicated in diabetes-induced tissue damage have been discussed by Brownlee [24, 25]. Downstream, it seems likely that these mechanisms converge on induction of TGF-β and CTGF. A schematic representation is shown in figure 3. Brownlee proposed that increased intracellular levels of glucose and fatty acids induce overproduction of reactive oxygen species (ROS), in particular superoxide, by the mitochondrial electron-transport chain. ROS is known to induce TGF-β as well as CTGF, and fibronectin gene expression [26]. Moreover, increased superoxide levels inhibit GAPDH activity, and the consequently decreased flux through the glycolysis pathway leads to activation of four important molecular mechanisms involved in the development of diabetic complications. These four molecular mechanisms are 1) increased flux through the polyol pathway, 2) increased formation of advanced glycation end products (AGEs), 3) activation of protein kinase C (PKC), and 4) increased flux through the hexosamine pathway.
1) Increased flux through the polyol pathway leads to a decreased NADPH/NADP+ ratio and thus a decrease in reduced glutathione, thereby diminishing the cellular antioxidant capacity. Together with the subsequent oxidation of sorbitol to fructose by sorbitol dehydrogenase, which generates ROS, this causes increased oxidative stress.
2) Diabetes leads to increased formation of AGEs, which alters the functional

properties of several important matrix molecules including collagen IV and laminin of basement membranes and affects also matrix-matrix and matrix-cell interaction. AGE proteins can also interact with specific receptors on the cellular surface and thus, via induction of ROS and NFkB, induce the expression of growth factors (including TGF-β and CTGF) and cytokines as well as the expression of pro-coagulatory and pro-inflammatory proteins. As for the kidney, AGEs have been described to induce TGF-β overexpression in glomerular mesangial cells. Furthermore, TGF-β independent upregulation of CTGF by AGEs has been described to occur through activation of the receptor for AGEs, RAGE [27, 28]. 3) The induction of PKC contributes to increased

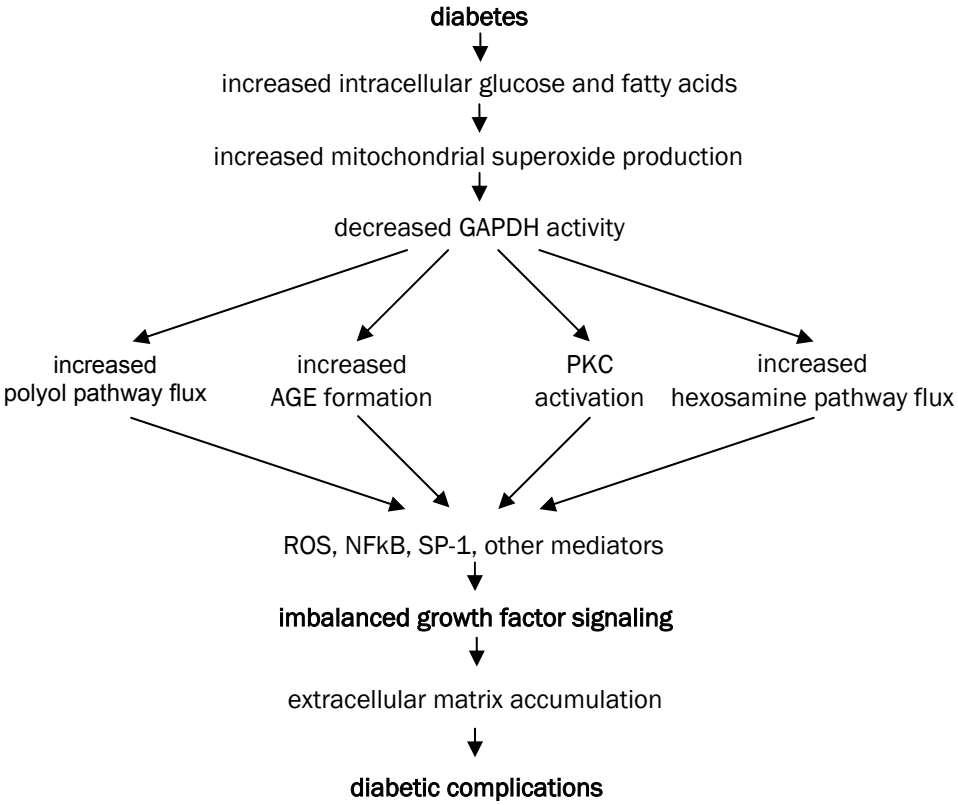

Figure 3: Schematic overview of the pathways involved in hyperglycemia-induced development of diabetic complications involving imbalance of growth factor signaling. Diabetes increases intracellular levels of glucose and fatty acids, which induce overproduction of superoxide by the mitochondrial electron-transport chain. Subsequently, increased superoxide levels inhibit GAPDH activity, and the consequently decreased flux through the glycolysis pathway leads to imbalanced growth factor signaling by the activation of four different molecular mechanisms. i.e. increased flux through the polyol pathway, increased formation of advanced glycation end products (AGEs), activation of protein kinase C (PKC), and increased flux through the hexosamine pathway. Together, this leads to imbalanced growth factor signaling, extracellular matrix accumulation and eventually to diabetic complications. Adapted from Brownlee [25].

mesangial matrix protein accumulation by induction of the expression of TGF-β, fibronectin and type IV collagen, both in cultured mesangial cells and in glomeruli of diabetic rats. Furthermore, PKC is involved in CTGF induction by TGF-β, as has been demonstrated in mesangial cells [29]. 4) Finally, increased flux of excess intracellular glucose into the hexosamine pathway, has been shown to increase the expression of TGF-β and plasminogen activator inhibitor (PAI)-1. Most likely, the increased expression of TGF-β will lead to (indirect) induction of CTGF as well. Induction of PAI-1 by the hexosamine pathway was shown to involve the transcription factor Sp-1 [30]. Although the CTGF promotor does contain an Sp-1 binding site which is involved in the constitutive overexpression of CTGF in scleroderma fibroblasts [22], direct effects of the hexosamine pathway on CTGF expression have not been reported so far.
Thus, by all of these pathways hyperglycemia can, induce the expression of growth factors. Although direct induction of CTGF has not been reported for each of these pathways, they are all known to induce TGF-β [31-35]. Since TGF-β is a major inducer of CTGF, it can be inferred that signaling through these pathways probably also leads to CTGF induction.

BIOLOGICAL FUNCTION OF CTGF

Biological effects of CTGF include stimulation of proliferation, angiogenesis, migration, ECM production, cell attachment, cell survival and in some cell types apoptosis [18]. These actions, however, are strongly dependent on the cell type and culturing conditions. Grotendorst et al. showed in renal fibroblasts *in vitro* that the two halves of CTGF have distinct biological effects and are able to function independent of each other [15]. The N-terminal domain mediates myofibroblast differentiation and collagen synthesis, while the C-terminal domain mediates fibroblast proliferation and DNA synthesis. Mediation of DNA synthesis was also reported previously for a 10 kD C-terminal fragment of CTGF [36]. Differentiation and increased collagen synthesis are mediated in concert with IGF via an IGF receptor, while cell proliferation and DNA synthesis are mediated in concert with EGF and EGF receptors [15]. This study indicated that full length CTGF is the most potent biologically active form of the protein in tissue culture and that selective degradation of one domain versus the other might function as a potential mechanism to select for proliferation or differentiation.
During development, CTGF is involved in chondrogenesis and endochondral ossification as was shown by its ability to promote proliferation, maturation and hypertrophy in articular cartilage cells *in vitro* [37]. CTGF knockout mice demonstrate skeletal defects including expanded hypertrophic zones of long bones, possibly due to decreases in growth plate angiogenesis, chondrocyte proliferation and matrix degrading enzymes [38]. Due to their skeletal malformations, which prevent proper breathing, CTGF knockout animals are not viable and die directly after birth [38].

High expression of CTGF was reported in the developing heart. However, morphological defects in the hearts of CTGF-deficient embryos were not detected, probably due to functional redundancy with other CCN family members [39].
CTGF is also involved in angiogenesis. CTGF promotes the adhesion, proliferation and migration of vascular endothelial cells and induces tube formation of these cells. Besides, CTGF was also able to induce a gross angiogenic response in the chicken chorioallantoic membrane [40]. In vivo, a strong angiogenic response was induced by injection of mice with collagen gel containing CTGF and by implantation of hydron pellets containing CTGF into rat corneas [40, 41]. In contrast, upon binding to CTGF, VEGF loses its ability to promote capillary tube formation in endothelial cell cultures (which was reversed with CTGF-neutralizing antibodies) and to promote angiogenesis when administered as a subcutaneous injection in Matrigel [9]. Therefore, it remains difficult to predict the net effect of CTGF (over)expression on angiogenesis and vascular maintenance.
CTGF expression is also involved in tumor progression, which might relate at least in part to its angiogenic effects. CTGF is elevated in tumors with a high level of neovascularization and increased CTGF was reported to be associated with decreased survival in patients with breast cancer [42], glioblastoma [43], or adenocarcinoma of the oesophagus [44], as well as with increased breast cancer bone metastasis in mice [45]. In contrast, high levels of CTGF were associated with better survival in patients with esophageal squamous cell carcinoma [44], and chondrosarcoma [46], and decreased metastasis in a mouse model of colon cancer [47].
Neovascularization of tumor cells *in vitro* can be inhibited by CTGF-neutralizing antibodies, which suggests that CTGF regulates progression of tumor angiogenesis, and that the release or secretion of CTGF from tumor cells is essential for the angiogenetic process [48]. Furthermore, treatment of mice bearing established CTGF-expressing pancreatic tumors, with CTGF-neutralizing antibodies abrogated CTGF-dependent tumor growth and inhibited lymph node metastases [49, 50]. In contrast, CTGF has been reported to induce apoptosis in both human breast cancer cells and human aortic smooth muscle cells *in vitro* [51, 52]. It thus appears, that depending on the cell type and environmental context, CTGF can exhibit both proliferative as well as anti-proliferative or apoptotic effects.
While CTGF expression in normal arteries is low, CTGF overexpression has been reported in atherosclerotic lesions, especially in complicated atherosclerotic plaques [17, 53]. Furthermore, in vitro studies have indicated that, by stimulation of chemotaxis, CTGF could contribute to recruitment of mononuclear cells to the artery wall [53], but how CTGF–monocyte interactions contribute to atherosclerosis still remains to be elucidated.

De novo synthesis of connective tissue occurs during the wound healing process [54]. Normally this response is appropriately terminated; however, if the wound healing process continues unabated, extracellular matrix (ECM) accumulates, and excess scar tissue is formed. Tissue fibrosis is generally considered to arise due to such failure of the normal wound healing response to terminate. In response to injury, mesenchymal fibroblasts become activated and proliferate and migrate into the wound where they synthesize elevated levels of ECM proteins [55]. These activated fibroblasts are also called myofibroblasts. They express α-smooth muscle actin (α-SMA) and have an enhanced ability to contract the extracellular matrix [56]. In fibrosis, the balance between ECM production and degradation is disturbed. CTGF is highly expressed in fibrotic lesions, and might also play an important role during physiological wound healing [57]. It is involved in the increased expression of ECM proteins [58] as well as in regulation of the expression and activity of MMPs and their inhibitor, tissue inhibitor of metalloproteinases-1 (TIMP-1) [59, 60]. The multiple interactions with matrix proteins, cell membrane proteins and growth factors make CTGF a multifunctional modulator of ECM remodeling in response to injury [1].

MECHANISM OF CTGF ACTION

Direct binding to cell surface receptors

No specific signaling receptor for CTGF has been identified thus far. However, CTGF has been reported to interact with several receptors previously implicated in signaling by other proteins. The first receptor shown to bind CTGF was the low-density lipoprotein receptor-related protein (LRP) [12]. LRP is normally expressed at low levels in the kidney but it is upregulated in glomeruli and tubulo-interstitium of rats with kidney fibrosis [61]. Tyrosine phosphorylation of this receptor upon binding of CTGF has been reported to be crucial for CTGF-induced myofibroblast differentiation [62]. In addition, CTGF interaction with the (Wnt co-receptor) LRP6 leads to inhibition of the Wnt signaling pathway [63]. The Wnt-signaling pathway is involved in embryonic development, tumor progression, and stem cell differentiation [64], and increased Wnt-dependent β-catenin signaling has been shown in a model of renal fibrosis in mice [65]. Furthermore, interaction of CTGF with, and subsequent activation of yet another receptor, the tyrosine kinase receptor TrkA, was shown to induce the transcription factor TGF-β inducible early gene (TIEG), which is critically involved in CTGF-dependent downregulation of Smad7 [66]. Since Smad7 acts as an inhibitor of pro-fibrotic TGF-β-signaling [67], its downregulation by CTGF-TrkA-induced TIEG might be an important aspect of the fibrogenic response.

Many of the effects of CTGF are thought to be mediated by binding to integrins, whereas for other effects its ability to interact with other growth factors may be more

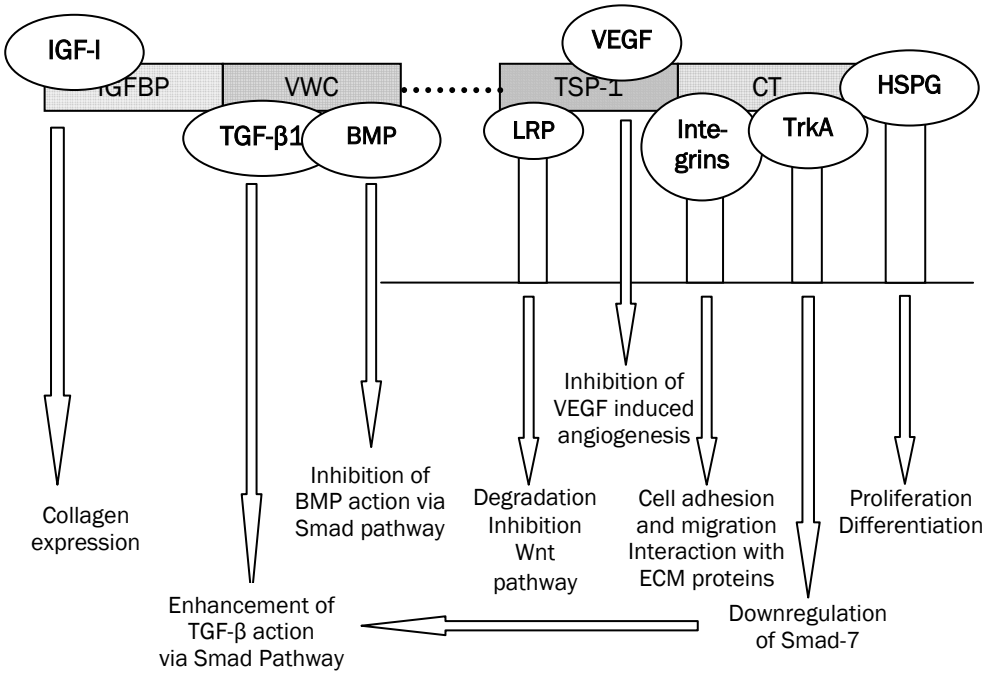

Figure 4: Biological effects of interaction of CTGF with growth factors and cell surface proteins.
Interaction of the IGFBP domain with IGF-I results in upregulation of collagen expression. By interaction of the VWC domain with TGF-β or BMP, CTGF modulates signaling through the Smad pathway. In addition interaction of CTGF, possibly via the CT domain, with TrkA influences the Smad pathway by downregulation of Smad7. Interaction of the TSP-1 domain with the LRP is involved in degradation of CTGF and might also inhibit the Wnt signaling pathway. By interacting with VEGF, CTGF inhibits VEGF-induced angiogenesis. Through its interaction with integrins as well as with HSPGs of the extracellular matrix, CTGF is involved in adhesion and migration.
IGFBP: insulin-like growth factor binding protein domain, VWC: von Willebrand factor type C domain, TSP-1: thrombospondin-1 domain, CT: C-terminal domain, IGF-I: insulin-like growth factor-I, TGF-β1: transforming growth factor-β1, BMP: bone morphogenetic protein, LRP: low-density lipoprotein receptor-related protein, VEGF: vascular endothelial growth factor, TrkA: tyrosine kinase A, HSPG: heparan sulphate proteoglycan.

important [6, 8, 9]. Integrins are heterodimeric transmembrane receptors that were first identified based on their ability to bind and mediate cell adhesion to distinct components of the extracellular matrix [68, 69]. Although the integrin family was initially identified based on the role of family members in mediating cell adhesion, it has become clear that integrins also play important roles in initiating biochemical signals that contribute to a wide variety of cellular responses, including survival, proliferation and migration [70]. In these responses, integrins function like other signaling receptors, initiating biochemical cascades of cytoplasmic protein modification

and translocation as a consequence of binding to extracellular ligands (outside-in signaling).
Studies in human mesangial cells showed that CTGF mediates fibronectin synthesis by upregulation of α5β1 integrin [71]. Interaction of CTGF with this integrin also enhances cell adhesion of chondrocytes in vitro [72], and mediates the adhesion and migration of pancreatic stellate cells [73]. Endothelial cell adhesion and migration is mediated by CTGF through integrin α5β3 [41], and binding of CTGF to this integrin (and HSPGs) also induces adhesion of rat activated hepatic stellate cells [13]. In addition, interaction of CTGF with α6β1 integrin stimulates collagen deposition by gingival fibroblasts [11]. Finally, integrins have been described to function as an adhesion receptor for CTGF to platelets and monocytes [74, 75], but the biological significance of this binding still remains to be established.
In vivo, diabetes induces aberrant integrin expression and localization in renal glomeruli [76-78]. In cultured human glomerular epithelial cells (HGEC), stimulation with high glucose resulted in decreased expression of α3-, α2-, and β1-integrins and increased expression of α5- and αvβ3 integrins. This change was accompanied by decreased binding of HGEC to type IV collagen [79].
As for binding of CTGF to HSPGs, this has been reported to regulate proliferation and differentiation of chondrocytes *in vitro* [14].

Modulation of the action of growth factors

Abreu et al., demonstrated that the cysteine-rich von Willebrand type C domain of CTGF can interact with TGF-β and BMP-4 [8]. They showed that TGF-β and BMP-4 compete to interact with this domain of the CTGF protein. Moreover, increasing amounts of CTGF antagonized BMP-4 binding to a recombinant type Ia BMP-receptor-Fc fusion protein while CTGF-TGF-β interaction increased binding of TGF-β1 to a TGF-β-receptor II-Fc recombinant protein.
So far, of the large family of BMPs, only binding of BMP-4 to CTGF has been reported while only BMP-7 has been addressed extensively as an anti-fibrotic cytokine in experimental renal disease including diabetic nephropathy [80-82]. However, it has been shown that BMP-4 as well as BMP-6 can substitute for BMP-7 loss during kidney development, which exemplifies that different BMP family members can function interchangeably to activate essential signaling pathways for growth and morphogenesis of the kidney [83]. Although this has not been resolved experimentally, it has been speculated that, like BMP-4 also BMP-7 signaling can be inhibited by CTGF. The possible interaction of CTGF with BMP-6 and BMP-7 is currently under investigation in our lab.
TGF-β and BMP both influence target gene expression through receptor mediated intracellular signaling via phosphorylation of Smad proteins. Binding of TGF-β to the

TGF-β type II receptor (TβRII) triggers the association of TβRII with a type I receptor (TβRI), which allows the constitutively phosphorylated TβRII to phosphorylate the TβRI. In most cases, the TβRI involved is the activin receptor-like kinase (ALK) 5. In the BMP signal transduction pathway, BMP usually first binds to the BMP type II receptor (BMPRII) but initial binding to the type I receptor (BMPRI) has also been reported. ALK2, ALK3 and ALK6 can all three function as BMPRI.

Once activated, type I receptors can recognize and phosphorylate pathway-specific receptor-regulated Smads (R-Smads) that reside in the cell cytoplasm. The "pro"-fibrotic pathway activated by TGF-β involves R-Smad 2 and 3 while the "anti"-fibrotic BMP pathway involves R-Smad 1, 5 and 8. Phosphorylation of the receptor-regulated Smad releases it from the receptor complex and allows it to associate with the co-Smad (Smad 4), which is common to the TGF-β and BMP signaling pathways. The R-Smad/Smad4 complex then translocates to the nucleus where it recognizes regulatory elements in target genes with the help of associated DNA-binding cofactors [84].

Smad binding elements (SBE) have been identified in the promotor region of numerous TGF-β induced genes. Genes subject to regulation by the Smad pathway include: CTGF [85], collagens [86, 87], MMPs [88], PAI-1 [89], Smad7 [90], α-smooth muscle actin (α-SMA) [91], and the PDGF-β chain [92].

Signaling by TGF-β1 via the Smad pathway can be increased by CTGF. At low TGF-β1 concentrations, CTGF potentiated the phosphorylation of Smad2 induced by TGF-β1 in fetal mink lung cell cultures. At higher concentrations of TGF-β1, CTGF did not increase Smad2 phosphorylation [8]. This indicates that CTGF serves mainly to potentiate the effects of limiting amounts of TGF-β1, and that it has itself no direct effect on Smad2 phosphorylation.

In conclusion, through binding to TGF-β and BMP, CTGF might modulate the equilibrium between the expression of pro-fibrotic and anti-fibrotic genes. In addition to interaction with TGF-β and BMP, CTGF can also interact with, and modulate the signaling by IGF-I and VEGF. IGF-I, is overexpressed together with CTGF in renal fibroblasts under diabetic conditions. Co-stimulation of CTGF and IGF-I in renal fibroblasts had a synergistic effect on the induction of collagen genes [7, 93]. VEGF was shown to bind to the third domain of CTGF, which resulted in reduced signaling activity of VEGF and consequent decrease of angiogenesis [9]. Of interest, cleavage of CTGF by MMPs leads to dissociation of the VEGF-CTGF complex and to restoration of the signaling activity of VEGF [5]. An overview of the effects of interaction of CTGF with the mentioned proteins is given in Figure 4.

These various interactions with cell surface proteins, extracellular matrix and other growth factors indicate that CTGF has important functions as a matricellular protein, modulating and integrating the role of other growth factors in the regulation of extracellular matrix.

THE ROLE OF CTGF IN DIABETIC NEPHROPATHY

Strong CTGF expression has been observed in glomeruli of diabetic patients and experimental diabetic animals with nephropathy [94-96]. Overexpression of CTGF was shown to be related to the severity of DN (chapter 5, [97]). In the light of what we know about its biological function and mechanism of action, this strongly suggests that CTGF may be a pathogenic (or progression) factor in DN. Moreover, CTGF expression was increased already in patients before they developed overt proteinuria [98]. Follow up data indicated that the progression to DN was possibly related to CTGF mRNA expression level [99], which suggested that CTGF, in addition to its proposed role in the pathogenesis and progression of DN, might also be useful as marker for DN.

CTGF as marker for diabetic nephropathy

Currently, (micro)albuminuria is still used as the major marker for development of DN. However, a significant percentage (55–70%) of diabetic patients with microalbuminuria will not progress to overt DN. Adequate therapy can even induce regression to normoalbuminuria in up to 30% of diabetic patients with microalbuminuria [100].

It has been proposed that CTGF might be a useful (additional) marker for development and progression of DN. Adler et al., showed in renal biopsies of diabetic patients that glomerular CTGF expression was upregulated in diabetic patients with microalbuminuria as well as overt nephropathy. In that study CTGF mRNA levels appeared to correlate with the degree of albuminuria [98]. The same study showed that the only normoalbuminuric patient with mRNA expression levels of CTGF and collagen above the 95% confidence interval of the normoalbuminuric group, was also the only patient who developed microalbuminuria at follow up [99].

Measurements of CTGF in plasma and urine samples of healthy control subjects as well as diabetic patients showed that the N-terminal half of the CTGF protein was readily detectable in all subjects and significantly elevated in diabetic patients. However, full length CTGF levels were below the detection limit of the ELISA in the majority of diabetic and non-diabetic plasma as well as urine samples tested (chapter 3, [101]). This absence of detectable full-length CTGF in plasma might be related to technical limitations (sensitivity of the full-length ELISA) or to its clearance via C-terminal interaction with matrix components and (scavenging) receptors, and to proteolysis, e.g. by MMPs, plasmin, and elastase, which have all been reported to cleave CTGF [5]. Both full length CTGF and N-terminal fragments are detectable in conditioned medium of mesangial cells in vitro. However, addition of full length recombinant human CTGF to mesangial cell cultures revealed that full length CTGF rapidly disappeared from the medium while the N-terminal fragment remained readily detectable (unpublished results). This might be explained either by binding and/or uptake by these cells, or by extracellular proteolysis of the CTGF protein.

In vitro, cell type and degree of confluency determine whether CTGF is secreted through the Golgi apparatus into the conditioned medium of cells [102, 103] or remains mainly attached to the ECM and the cell surface [36].
Recently, plasma CTGF levels and urinary CTGF excretion have been studied in diabetic patients. A preliminary study by Riser et al. reported that urinary CTGF excretion is low in healthy volunteers as well as in most diabetic patients without kidney disease, while it is markedly increased in the majority of diabetic patients with DN [104]. Subsequently, Gilbert and co-workers performed a study in 31 type 1 diabetic patients and showed that urinary CTGF excretion was about 10 times increased in microalbuminuric patients, while it was about 100 times increased in patients with overt DN. In this study, urinary CTGF excretion was closely correlated to the degree of albuminuria [105]. The latter is in agreement with the observations of the so far only large study of urinary CTGF excretion in type 1 diabetic patients and healthy control subjects, which was conducted in our lab [106]. We observed that urinary CTGF excretion was significantly increased in patients with overt DN, compared to normoalbuminuric and microalbuminuric patients, as well as to healthy controls. In contrast, to the study by Gilbert and co-workers, our study revealed a mean increase of urinary CTGF excretion only 1.5 times higher than normal in a group of 89 patients with DN, and no increase at all in microalbuminuric patients. Within the group of patients with DN, CTGF correlated with urinary albumin excretion as well as GFR. However, we found no differences in urinary CTGF excretion between normoalbuminuric and microalbuminuric patients.
In a small study of type 1 diabetic patients with normoalbuminuria, microalbuminuria or DN as well as healthy volunteers, we observed that plasma levels of CTGF are significantly increased in patients with DN (chapter 3, [101]). In addition we found that plasma CTGF levels were also correlated with markers for nephropathy, i.e. with both albuminuria and creatinine clearance. A very wide variation in plasma CTGF level was observed in the group of microalbuminuric patients. Follow up data on microalbuminuric patients with and without high plasma CTGF levels will be essential for assessment of the predictive value thereof with respect to later development of DN.
In a subsequent, large study of patients with type 1 diabetes with and without nephropathy, we observed that a standardized increase of plasma CTGF level with 1 SD was associated with an equally strong increase of the risk for DN as a 1 SD increase of HbA_{1c} and systolic blood pressure, which are considered the main risk factors for development of DN. Strikingly, this relative risk is also equal to that inferred by a 1 SD elevation of urinary CTGF excretion [106]. In addition, we found that, within the group of patient with DN, plasma CTGF levels are indeed correlated with renal function, while in normoalbuminuric patients no such correlation was found (Roestenberg *submitted*, chapter 4).

In a separate study, angiotensin II receptor blockade in hypertensive type 1 diabetic patients with DN significantly reduced urinary CTGF excretion, while plasma CTGF levels remained unchanged [107]. Moreover, the reduction of urinary CTGF excretion correlated with a lower rate of GFR-decline in these patients.
Thus, in type 1 diabetic patients with nephropathy, increased CTGF mRNA as well as plasma and urinary CTGF levels are associated with loss of renal function and proteinuria, both markers of DN. Unfortunately, all studies available thus far, have only analyzed transversal, retrospective data, and plasma and urine CTGF levels have not been available for the same patients. Due to these limitations, it is still difficult to determine the value of plasma- and urinary CTGF as a marker for development and progression of DN.

CTGF as pathogenic factor in diabetic nephropathy

CTGF expression in renal cells is increased by high glucose, AGEs, TGF-β and mechanical strain, which are all considered to contribute to diabetic nephropathy. In addition, CTGF is well known to induce the (over)expression of ECM proteins *in vitro* [18, 108-110].
The involvement of CTGF in renal fibrosis *in vivo* was identified in different animal models of fibrotic kidney disease: In a rat model of renal insufficiency, 5/6 nephrectomy, it has been shown that administration of fluvastatin significantly prevented the renal dysfunction and histological abnormalities in combination with reduction of CTGF levels. Furthermore, both significant suppression of MMP-activity and significant activation of TIMPs were almost completely prevented by fluvastatin, which resulted in a significant reduction of glomerular ECM accumulation [111].
In both type 1 and type 2 diabetic mice, we showed that renal CTGF expression as well as plasma- and urinary CTGF levels are increased early in the disease. CTGF mRNA and protein expression was upregulated most prominently in the kidney, in particular in podocytes. Furthermore, urinary CTGF levels in diabetic mice were strongly correlated with albuminuria (chapter 5, [97]). This temporal profile and distribution pattern suggest that CTGF is involved in development and progression of DN in these models of experimental type 1 and 2 diabetes.
Also in diabetic patients, we observed that plasma CTGF level and urinary CTGF excretion correlated with parameters for DN (chapter 3, [101] and [106]). Moreover, treatment of diabetic patients with the angiotensin II receptor blocker Losartan resulted in reduced urinary CTGF levels which was accompanied by a reduction of the decrease of GFR in these patients [107].
In a rat model of experimental diabetic nephropathy, aminoguanidine, an inhibitor of advanced glycation, prevented the expression of CTGF, which was associated with reduced accumulation of fibronectin [112]. Another study revealed that glomerular

expression of CTGF and TGF-β1 mRNA was coordinately upregulated as early as day 3 after induction of DM in rats, followed by increased fibronectin expression and mesangial matrix accumulation [113]. Chronic aspirin treatment significantly attenuated mesangial expansion, and effectively suppressed the induction of CTGF, as well as the upregulation of TGF-β1 and fibronectin expression.
To investigate the importance of the level of CTGF expression for development of DN, we induced diabetes in $CTGF^{+/-}$ mice, lacking one functional CTGF allele, and in their wild type $CTGF^{+/+}$ littermates. In diabetic $CTGF^{+/-}$ mice, CTGF levels were significantly lower than in diabetic $CTGF^{+/+}$ littermates. In addition, diabetic $CTGF^{+/-}$ mice had significantly less albuminuria and totally lacked GBM thickening. This was associated with preserved MMP-activity (Roestenberg submitted, chapter 6). Similar observations have been reported in db/db mice treated with CTGF-neutralizing antibodies, which resulted in prevention of GBM thickening and attenuation of several parameters of renal disease, including albuminuria [114].
Together, these data indicate that CTGF is an important pathogenic factor in DN.

CTGF as target for therapeutic intervention of diabetic nephropathy

TGF-β is generally regarded as the main pro-fibrotic growth factor in DN. However, due to its anti-inflammatory and anti-proliferative effects, inhibition of TGF-β as treatment of fibrotic disease might carry significant risks. The important role of TGF-β1 in immune regulation is exemplified by TGF-β1 knockout mice, which die at an age of 3-4 weeks due to a hyperinflammatory disease in almost all tissues [115]. CTGF is a downstream mediator of the fibrotic effects of TGF-β, and it seems likely that CTGF might be a more suitable target for intervention in DN and other fibrotic diseases [108-110]. Although CTGF was also shown to have some anti-proliferative effects on certain cell types, no immune regulatory role has been described for CTGF to date. The importance of CTGF for maintaining homeostasis and the possible side-effects of CTGF-inhibition in treatment of fibrotic diseases will be an important subject of future studies.
Several studies addressed the potential of CTGF inhibition to attenuate renal fibrosis. *In vitro*, transfection of renal fibroblasts with antisense CTGF blocked TGF-β-stimulated collagen synthesis [116]. In addition, transfection of CTGF antisense oligonucleotides (ODN) in cultured renal fibroblasts significantly attenuated TGF-β-stimulated upregulation of fibronectin [117]. Blockade of CTGF under diabetic conditions has been studied in several different renal cell types *in vitro*. An CTGF-neutralizing antibody partly inhibited the high-glucose-induced collagen production in human renal fibroblasts [7]. In addition, the high-glucose-induced increase of the synthesis of fibronectin and PAI-1 in human mesangial cell cultures was inhibited by CTGF antisense ODN treatment [118]. Moreover, CTGF antisense ODN also attenuated angiotensin II and AGE-induced collagen I production in renal interstitial fibroblasts [119]. Transfection of CTGF

antisense ODNs in cultured human mesangial cells showed that CTGF is required for synthesis of fibronectin in response to stimulation by high glucose or glycated albumin [120]. In addition, CTGF-neutralizing antibodies also attenuated the high-glucose- or TGF-β-induced inhibition of matrix degradation in human mesangial cells [60].

The number of *in vivo* studies of the effect of CTGF blockade on fibrosis and especially DN is still limited, although it does include several interesting models. Recently, silencing of CTGF by means of siRNA markedly, but not completely, inhibited ECM production and deposition in a rat model for liver fibrosis [121]. In addition, the latter study showed that CTGF siRNA prevented the upregulation of TGF-β1 activity and protein expression in fibrotic rat liver. How silencing of CTGF inhibits the upregulation of TGF-β1 is as yet poorly defined. The fact that CTGF siRNA did not affect the expression of Smad (-2 and) -7 gene in the latter study [121], suggests a less prominent role of the TrkA-TIEG pathway in mediation of CTGF effects (at least) in this particular model of liver fibrosis. In another model, rats with a unilateral ureter obstruction (UUO) were treated with CTGF antisense ODN [117]. This treatment markedly attenuated the induction of CTGF, fibronectin, fibronectin ED-A, and collagen Iα1 genes, whereas TGF-β gene upregulation was not affected. The antisense ODN treatment also reduced interstitial deposition of CTGF, fibronectin ED-A, and type I collagen. Also the size of interstitial fibrotic areas, and the number of myofibroblasts (determined by the expression of α-SMA) was significantly decreased in CTGF antisense ODN treated rats [117]. In the remnant kidney of subtotally nephrectomized TGF-β1 transgenic mice, intravenous administration of CTGF antisense ODN blocked CTGF expression in proximal tubular epithelial cells despite the sustained high level of TGF-β1 mRNA. This reduction of CTGF mRNA level was paralleled by a reduction of mRNA levels of matrix molecules as well as proteinase inhibitors PAI-1 and TIMP-1, and by suppressed renal interstitial fibrogenesis [122].

As was mentioned before, genetic prevention of the upregulation of CTGF expression under diabetic conditions decreased albuminuria, and also prevented GBM thickening and decrease of the gelatinase activity of MMPs (Roestenberg, chapter 6 *submitted*). A similar attenuation of DN was obtained by treatment of db/db mice with CTGF-neutralizing antibodies [114]. In a recent phase 1 trial, a CTGF-neutralizing antibody was found to be safe and well tolerated in patients suffering from idiopathic pulmonary fibrosis [123]. Currently, diabetic patients with microalbuminuria are being recruited for a phase 1b trial with the same antibody, in preparation for future studies into the efficacy of anti-CTGF therapy for prevention and treatment of incipient and established DN.

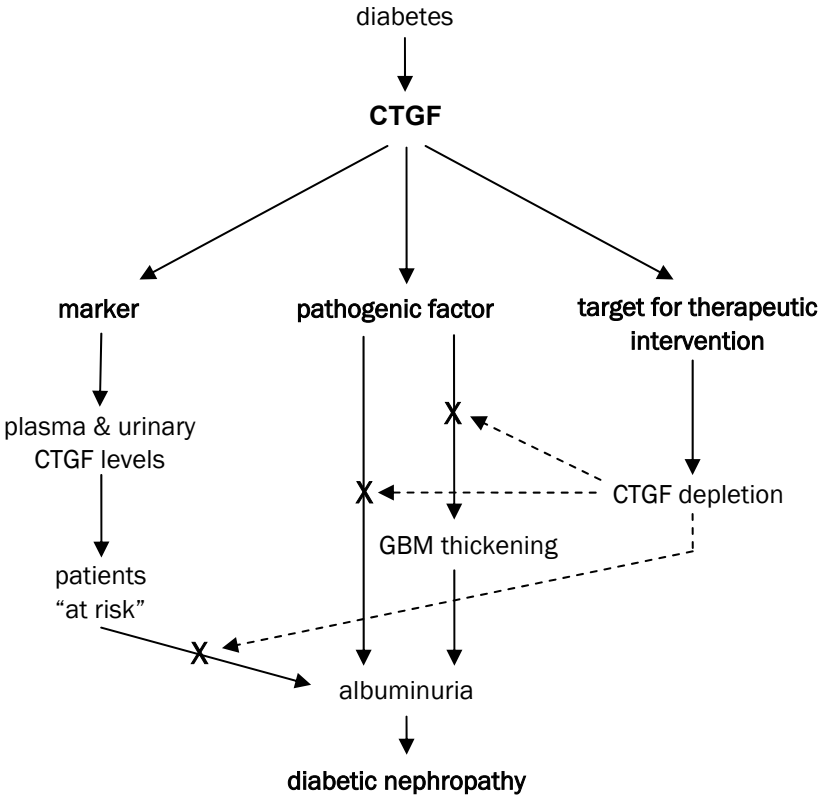

Figure 5: The possible roles of CTGF in diabetic nephropathy: marker, pathogenic factor and target for therapeutic intervention.

In conclusion; CTGF certainly appears to play a pivotal role in development and progression of DN. It has potential for use as clinical marker and target for prevention and probably also treatment of diabetic nephropathy (figure 5). Further investigations are needed to elucidate the precise mechanism of action of CTGF in DN.

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Chapter 3

Connective tissue growth factor is increased in plasma of type 1 diabetic patients with nephropathy

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ABSTRACT

OBJECTIVE—Connective tissue growth factor (CTGF) is strongly upregulated in fibrotic disorders and has been hypothesized to play a role in the development and progression of diabetes complications. The aim of the present study was to investigate the possible association of plasma CTGF levels in type 1 diabetic patients with markers relevant to development of diabetes complications.

RESEARCH DESIGN AND METHODS—Plasma CTGF levels (full-length and NH_2-terminal fragments) were determined in 62 well-characterized patients with type 1 diabetes and in 21 healthy control subjects. Correlations of these plasma CTGF levels with markers of glycemic control, platelet activation, endothelial activation, nephropathy, and retinopathy were investigated.

RESULTS—Elevated plasma NH_2-terminal fragment of CTGF (CTGF-N) levels were detected in a subpopulation of type 1 diabetic patients and were associated with diabetic nephropathy. Stepwise regression analysis revealed contribution of albuminuria, creatinine clearance, and duration of diabetes as predictors of plasma CTGF-N level. Elevation of plasma CTGF-N levels in patients with retinopathy was probably due to renal co-morbidity.

CONCLUSIONS—Plasma CTGF-N levels are elevated in type 1 diabetic patients with nephropathy and appear to be correlated with proteinuria and creatinine clearance. Further studies will be needed to determine the relevance of plasma CTGF as a clinical marker and/or pathogenic factor in diabetic nephropathy.

INTRODUCTION

Diabetic patients frequently develop severe chronic complications like cardiovascular disease, nephropathy, neuropathy, and retinopathy. Characteristics of these complications are macro- and microvascular damage, extracellular matrix (ECM) accumulation, and eventually chronic fibrosis. Growth factors play an important role in the development of these diabetes complications [1-4]. One of the important growth factors involved in ECM accumulation and fibrotic processes is transforming growth factor (TGF)-β [1]. In both type 1 and type 2 diabetes, plasma levels of TGF-β were correlated with the presence of diabetes complications [5, 6].

Connective tissue growth factor (CTGF) is another important growth factor implicated in the development of diabetes complications. CTGF is a 38-kDa, cystein-rich secreted protein that was originally cloned from human umbilical vein endothelial cells [7]. Strong CTGF expression has been reported [8, 9] in atherosclerotic aorta and in renal mesangial cells cultured in high-glucose medium. CTGF was also shown [10-12] to be strongly expressed in glomeruli of diabetic patients and animals with nephropathy. Similarly, the magnitude of urinary CTGF excretion was related to the severity of diabetic nephropathy (DN) in a cross-sectional study [13] of patients with type 1 diabetes. Both high glucose concentrations and advanced glycation end products (AGEs) are able to induce ECM production via CTGF [8, 14]. CTGF itself can act as a downstream mediator of TGF-β in ECM synthesis, but TGF-β–independent regulation of CTGF has also been reported [8, 15]. Therefore, the analysis of CTGF plasma levels of diabetic patients might provide important additional information about the involvement of this growth factor in the development of diabetes complications. Different fragments of the CTGF protein have been detected in vitro and in vivo, and at least some of these have biological activity. The CTGF molecule, which consists of four modules, is mostly cleaved between modules II and III, yielding fragments of 16–20 kDa, but smaller fragments have also been identified [16, 17]. The relative contribution to fibrotic processes of full-length compared with fragmented CTGF remains to be established.

The aim of the present study was to investigate the possible association of plasma CTGF levels in 62 well-characterized type 1 diabetic patients with DN and diabetic retinopathy as well as with general patient characteristics and other markers relevant to the development of diabetes complications, i.e., glycemic control, endothelial activation, and platelet activation [4, 18-20].

RESEARCH DESIGN AND METHODS

For the present study, we analyzed plasma samples obtained from 62 well-characterized patients with type 1 diabetes and 21 healthy control subjects. All gave their informed consent before participation in the study. The study protocol has been approved by the local ethics committee/institutional board and was conducted according to the principles of the Declaration of Helsinki. After an overnight fast and abstaining from vigorous physical activity during the previous 24 h, patients and control subjects presented at the outpatient clinic between 8:00 and 10:00 A.M., bringing their 24-h urine. Demographic and relevant medical history data were recorded, including age, sex, duration of diabetes, insulin dose, co-morbidity, medication, and smoking habits. Blood pressure was measured with a sphygmomanometer in the sitting position; the median of three successive measurements was noted. Height and weight were measured to determine BMI. Retinopathy was scored by a single experienced ophthalmologist by fundoscopic examination and examination of the clinical charts.

Blood was collected by puncture of an antebrachial vein using sodium heparin, citrate, or PECT as anticoagulants, dependent on the assay. PECT medium (400 µl/4.5 ml polypropylene tube), used to prevent ex vivo platelet activation, is composed of equal volumes of solutions A, B, and C, in which A = 282 nmol/l prostaglandin E1 and 1.9 mmol/l Na2CO3, B = 30 mmol/l theophylline in PBS, and C = 270 mmol/l EDTA. To further avoid artificial platelet and leukocyte activation, blood samples were immediately cooled on ice and platelets were depleted by centrifuging the samples for 60 min at 4,000g and 4°C within 1 h after collection. All plasma samples were stored at –70°C until analysis.

Measurement of markers for glycemic control and nephropathy

Concentrations of HbA_{1c} were determined in citrate plasma by means of high-performance liquid chromatography. Plasma creatinine concentrations were determined by automated spectrophotometrical assay using Creatinine-PAP (peroxidase-antiperoxidase). In 24-h urine samples, creatinine was measured using the Jaffé method, albumin was determined with an immunonephelometric assay, and renal creatinine clearance was calculated from these data.

Measurement of markers for platelet and endothelial activation

Von Willebrand factor (vWF) antigen was determined by sandwich enzyme-linked immunosorbent assay (ELISA) using rabbit anti-human vWF as "capture" antibody and horseradish peroxidase–conjugated rabbit anti-human vWF as "detecting" antibody (Dakopatts, Glostrup, Denmark). β-Thromboglobulin, a marker for in vivo platelet activation, and platelet factor 4, a marker for ex vivo platelet activation, were

determined in platelet-depleted PECT plasma by means of sandwich ELISAs according to the manufacturer's instructions (Asserachrom; Diagnostica Stago) [21].

ELISAs for plasma CTGF (NH_2-terminal and full length) and TGF-β

Plasma content of full-length and N-terminal fragments (CTGF-N) of CTGF were determined by means of two separate sandwich ELISAs, each using two distinct monoclonal antibodies against human CTGF (FibroGen, South San Francisco, CA). Microtiter plates were coated overnight at 4°C with capture antibody and blocked with BSA. Citrate plasma was diluted 5- or 10-fold in assay buffer, and 50-µl diluted sample was added to each well together with 50 µl biotinylated CTGF detection antibody. After incubation for 2 h at 37°C followed by incubation with streptavidin-conjugated alkaline phosphatase for 1 h at room temperature, plates were washed and 100 µl of substrate solution containing p-nitrophenyl phosphate was added to each well. After 20 min of color development, absorbance was read at 405 nm. Purified recombinant human CTGF (FibroGen) was used for calibration. Both monoclonal antibodies used in the CTGF-N sandwich ELISA specifically bind distinct epitopes on the N-terminal half of the CTGF protein. This assay detects both CTGF-N as well as the full-length CTGF protein. In the full-length CTGF ELISA, the capture antibody binds the COOH-terminal part of the CTGF protein, whereas the detecting antibody (the same as in the CTGF-N ELISA) binds the NH_2-terminal part of the CTGF protein. To avoid confusion due to differences in the molecular weight of full-length and different fragments of CTGF, all levels were expressed as picomoles per liter. The detection limit of these assays was 4 pmol/l for CTGF-N and 8 pmol/l for full-length CTGF, and intra- and interassay variations were 6 and 20%, respectively.

TGF-β1 was determined in PECT plasma by means of sandwich ELISA according to the manufacturer's protocol (R&D Systems, Minneapolis, MN).

Statistical analysis

Data are expressed as mean ± SD. Mann-Whitney analysis and Kruskal-Wallis analysis followed by Dunn's method were performed to determine differences in plasma CTGF-N levels between groups. Forward stepwise regression analysis was used to compare CTGF-N levels with general patient characteristics, glycemic control, endothelial activation, platelet activation, albuminuria, and creatinine clearance. Because data were not normally distributed, Spearman's correlations were calculated between CTGF-N levels and HbA_{1c}, albuminuria, and TGF-β1 levels. In all cases, $P < 0.05$ was considered significant.

RESULTS

Plasma CTGF-N level is elevated in DN

Levels of full-length CTGF were below the sensitivity limit of our sandwich ELISA in all control as well as diabetic plasma samples tested, although full-length recombinant CTGF was readily detectable if spiked in these plasma samples. This means that these plasma samples contained <80 pmol/l of full-length CTGF (not shown). In contrast, CTGF-N was readily detectable in the majority of these same plasma samples. No significant difference was found in CTGF-N levels between control subjects and the total group of diabetic patients, but the variation was much larger among samples from diabetic patients (138 ± 136 pmol/l in diabetic patients vs. 103 ± 51 pmol/l in control subjects) (Fig. 1A). Patients were divided into subgroups according to the level of albuminuria, i.e., normoalbuminuria (<30 mg/day), microalbuminuria (30–300 mg/day), and overt proteinuria (i.e., DN, >300 mg/day). This revealed significant differences in plasma CTGF-N levels between DN patients and control subjects and between DN and normoalbuminuric patients (Fig. 1B). Furthermore, a very wide variation in plasma CTGF-N levels was noted within the subgroup of microalbuminuric patients (203 ± 203 pmol/l).

Plasma CTGF-N level correlates with HbA_{1c}

General patient characteristics and markers of glycemic control, endothelial activation, platelet activation, and nephropathy of healthy subjects and normoalbuminuric, microalbuminuric, and DN diabetic patients are summarized in Table 1.

HbA_{1c} levels in the diabetic patients correlated, albeit only weakly, with plasma CTGF-N ($R = 0.355$, $P = 0.005$) (Fig. 1C). This is in line with induction of CTGF expression by high ambient glucose and AGEs observed previously in vitro [8, 14]. No significant correlation between plasma CTGF-N and HbA_{1c} was found within any of the subgroups. This might be due to the relatively small numbers of microalbuminuric and DN patients.

Plasma CTGF-N correlates with albuminuria and creatinine clearance

Forward stepwise regression analysis showed that albuminuria, creatinine clearance, and duration of diabetes are independent predictors of plasma CTGF-N level. Plasma CTGF-N most strongly correlated with albuminuria ($R = 0.572$, $P < 0.001$) (Fig. 1D), whereas albuminuria and creatinine clearance together yielded a cumulative R of 0.667, with $P < 0.001$. Correlation of CTGF-N with albuminuria, creatinine clearance, and duration of the diabetes together resulted in a cumulative R of 0.759, with a $P = 0.001$. Addition of further parameters did not significantly contribute to this correlation. Within the group of DN patients, the plasma CTGF-N levels showed a tendency to correlate with albuminuria, but this was not statistically significant ($n = 10$, $R = 0.553$,

	Control subjects	Type 1 diabetic patients		
		NA	MA	DN
General patient characteristics				
n (men)	21 (11)	40 (19)	12 (6)	10 (6)
Age (years)	30 ± 6	37 ± 10	45 ± 9	43 ± 10
Duration of diabetes (years)	—	18.3 ± 12.0	23.0 ± 8.8	26.2 ± 6.2
BMI (kg/m^2)	23.4 ± 3.3	23.2 ± 2.7	22.0 ± 2.0	22.6 ± 2.1
Systolic blood pressure (mmHg)	129 ± 15	132 ± 18	141 ± 18	152 ± 22
Glycemic control				
HbA_{1c} (%)	5.2 ± 0.4	8.0 ± 1.7	8.3 ± 1.1	9.2 ± 1.2
Platelet activation				
β-Thromboglobulin (units/l)	28.5 ± 8.8	27.8 ± 6.4	33.4 ± 6.5	34.6 ± 9.9
Endothelial activation				
vWF (%)	78 ± 18	103 ± 31	111 ± 23	129 ± 43
Nephropathy				
Albuminuria (mg/24 h)	9.2 ± 7.4	8.9 ± 5.1	102 ± 82	953 ± 690
Creatinine clearance (ml/min)	137 ± 22	131 ± 37	120 ± 46	87 ± 34

Table 1: General and clinical parameters of healthy control subjects and type 1 diabetic patients
Data are means ± SD. MA, microalbuminuric; NA, normoalbuminuric.

P = 0.097). When, instead of plasma CTGF-N level, albuminuria or creatinine clearance was taken as a dependent variable in the forward stepwise regression analysis of this dataset, plasma CTGF-N level was identified as the strongest independent predictor (R = 0.572 and 0.514, respectively). It thus appears that plasma CTGF-N levels are correlated with markers for nephropathy.

Plasma TGF-β1 levels

Since TGF-β1, unlike CTGF (F.A.V.N., P.R., unpublished observations) is abundant in platelets and released during platelet activation, reliable estimates of "in vivo" plasma TGF-β1 levels can be obtained only in the absence of significant ex vivo platelet activation (i.e., platelet factor 4 <10 units/l). Despite careful plasma collection and processing, only 21 diabetic and 5 control samples met this criterion. In these 26 samples, TGF-β1 levels did correlate with CTGF-N levels (R = 0.671, P < 0.001), but no

significant difference in plasma TGF-β1 levels was observed between healthy subjects and diabetic patients. Within the group of 21 diabetic patients, TGF-β1 correlated with HbA_{1c} (R = 0.477, P = 0.029). This is stronger than the correlation between plasma CTGF-N and HbA_{1c}.
In subgroups defined by degree of albuminuria, no significant difference was found between TGF-β1 levels, although there seemed to be a trend to higher levels in patients with DN as compared with diabetic patients without DN (P = 0.114) (data not shown). Due to small numbers, it was not possible to include TGF-β1 as a parameter in forward stepwise regression analysis.

Plasma CTGF-N levels in patients with retinopathy

Diabetic retinopathy is almost invariably present in patients with DN. Plasma CTGF-N levels appeared to be significantly elevated in patients with retinopathy compared with those without retinopathy (P < 0.001) (Fig. 1E). However, of the 17 patients with diabetic retinopathy who had >180 pmol/l of CTGF-N in their plasma (upper limit of normal control subjects), only 4 were normoalbuminuric, whereas 4 of the remaining 13 were microalbuminuric and 9 proteinuric (i.e., had DN). Plasma CTGF-N levels of normoalbuminuric patients with retinopathy (n = 23) were not different from those of normoalbuminuric patients without retinopathy (n = 17).

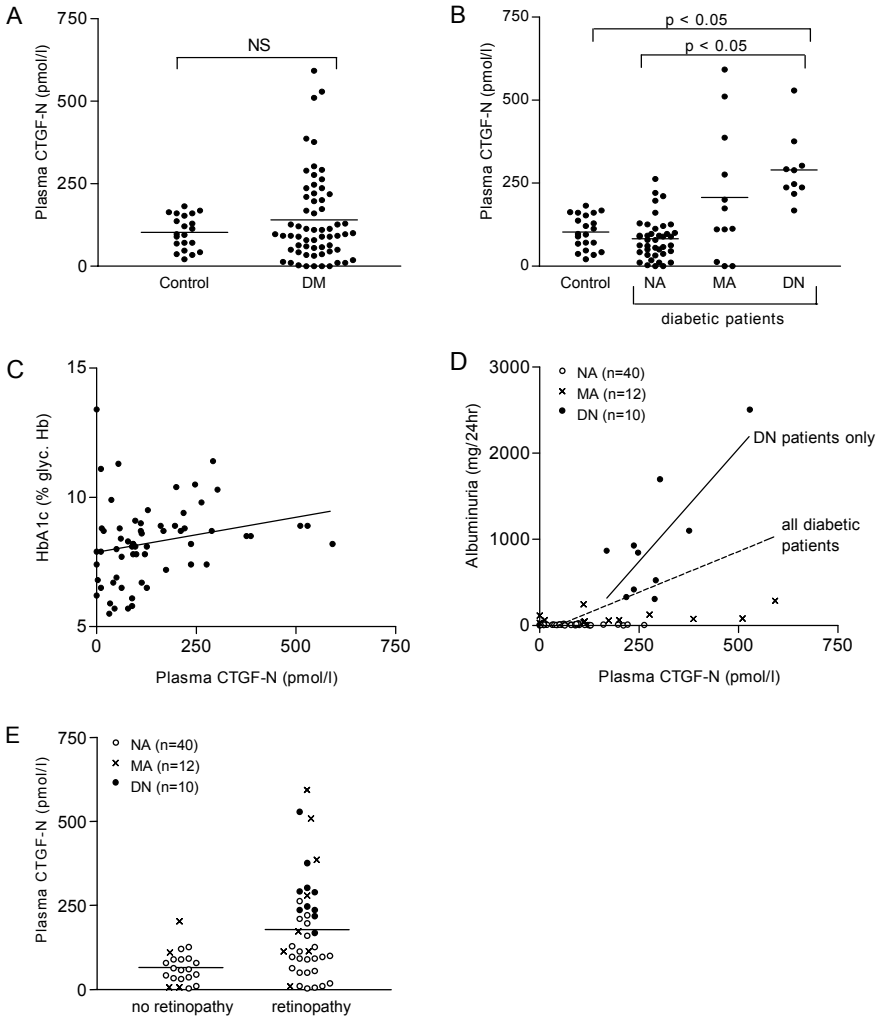

Figure 1: Plasma CTGF-N levels and correlations in control subjects and different subgroups of type 1 diabetic patients. A: Plasma CTGF-N levels in healthy control subjects (103 ± 51 pmol/l [mean ± SD]) and all diabetic patients (138 ± 136 pmol/l) (P = 0.765). B: Distribution of plasma CTGF-N fragment levels in the different patient subgroups according to albuminuria: healthy control subjects, 103 ± 51 pmol/l; normoalbuminuric (NA), 80 ± 66; microalbuminuric (MA), 203 ± 203; and DN, 290 ± 101 (mean ± SD). A significant difference (P < 0.05) was observed between control subjects and DN patients and between normoalbuminuric and DN patients. C: Correlation between plasma CTGF-N levels and HbA_{1c} in all diabetic patients. Spearman's correlation: R = 0.355, P = 0.005. D: Correlations between plasma CTGF-N and albuminuria in the total group of type 1 diabetic patients and in the subgroup of DN patients only. Diabetic patients (n = 62): R = 0.572, P < 0.001. DN patients only (Spearman's correlation, n = 10): R = 0.553, P = 0.097. E: Distribution of plasma CTGF-N levels in diabetic patients with and without retinopathy: no retinopathy, 61 ± 57 pmol/l, and retinopathy, 178 ± 148 (mean ± SD) (P < 0.001). Elevated plasma CTGF-N levels are almost exclusively found in microalbuminuric and DN patients. ○ normoalbuminuric; x microalbuminuric; ●, DN.

CONCLUSIONS

Changes in growth factor balance are important in the development of chronic complications of diabetes. In this study, we observed that plasma CTGF-N levels are significantly elevated in patients with DN and correlated with markers for DN and glycemic control. Full-length CTGF levels were below the detection limit of our ELISA in all diabetic and normal plasma samples tested. This absence of detectable full-length CTGF in plasma might be related to technical limitations (sensitivity of the full-length ELISA) or to its clearance via COOH-terminal interaction with matrix components and (scavenging) receptors and to proteolysis, e.g., by matrix metalloproteinases, plasmin, and elastase, which have all been reported to cleave CTGF [17].

It has been reported [10-13, 22] that CTGF mRNA and protein levels are significantly increased in kidney tissue and urine of patients as well as experimental animals with DN. Adler et al. [23] found equally elevated CTGF mRNA levels in glomeruli of microalbuminuric (n = 5) and DN (n = 6) diabetic patients. We now add to this notion that CTGF-N levels are elevated in plasma of almost all type 1 diabetic patients with DN and also in about one-third of microalbuminuric patients, but only in a small minority of normoalbuminuric patients. Plasma CTGF-N levels of patients with DN as a group differed significantly from those of normoalbuminuric patients and healthy control subjects. Due to very wide scatter, levels in microalbuminuric patients were not significantly different from those in healthy control subjects or normoalbuminuric patients and also not significantly different from levels in patients with DN. Since there was a difference in age between healthy control subjects and microalbuminuric and DN diabetic patients, we also measured plasma CTGF-N in an additional group of 20 healthy subjects whose mean age (42 ± 8 years) was comparable with that of the studied microalbuminuric and DN diabetic patients. In this additional control group, we found a mean plasma CTGF-N level of 120 ± 72 pmol/l, which is in the same range as the plasma CTGF-N levels in the other healthy control group and the normoalbuminuric diabetic patient group. It was not possible to include these additional 20 control subjects in the study because other parameters were not determined in these individuals.

The apparent discrepancy with the similarly elevated kidney mRNA levels in all five microalbuminuric patients and six patients with DN reported by Adler et al. [23] might indicate that plasma CTGF level is not solely determined by renal CTGF expression. Moreover, microalbuminuric patients are heterogeneous with respect to progression. A recent meta-analysis by Caramori et al. [24] revealed a 30–45% risk of progression of microalbuminuria to proteinuria over 10 years compared with 30% remission to a normoalbuminuric state and stabilization of microalbuminuria in the remaining patients. In this respect, it is of interest that our microalbuminuric group showed a remarkable interindividual variation in plasma CTGF-N levels, with significantly elevated

levels in 4 of 12 microalbuminuric patients. Obviously, it will be important to obtain follow-up data of these and other microalbuminuric diabetic patients to investigate whether high plasma CTGF-N levels might predict progression of microalbuminuria to DN.

The major finding in forward stepwise regression analysis was that plasma CTGF-N levels were associated with albuminuria, creatinine clearance, and duration of diabetes. The strongest correlation was found with albuminuria, one of the main characteristics of DN, and a negative but almost equally strong correlation was found with creatinine clearance. Identical results were obtained when the MDRD (modification of diet in renal disease) formula and Cockcroft-Gault calculations were used as estimates of glomerular filtration rate instead of creatinine clearance. CTGF (36–38 kDa), and even more its fragments (~10–20 kDa), can be expected to pass the glomerular filter into the primary urine, although dimerization and other protein interactions might prevent filtration in normal glomeruli. In DN, proteinuria is accompanied by a progressive decline of glomerular filtration. Therefore, elevated plasma CTGF-N levels in patients with DN might (at least in part) reflect loss of renal clearance. In conjunction with albuminuria and creatinine clearance, duration of diabetes was identified as a third factor that significantly contributed to plasma CTGF-N levels. No co-linearity between albuminuria, creatinine clearance, and duration of diabetes was observed, which indicates that in this analysis these three parameters can be considered as independent factors. Nevertheless, a certain degree of interdependence might be assumed because DN tends to develop about 10–15 years after the onset of type 1 diabetes, starting with microalbuminuria, followed by proteinuria and later progressive loss of renal function.

Although CTGF has originally been cloned from endothelial cells and atherosclerotic aorta [7, 9], markers for endothelial or platelet activation (vWF and β-thromboglobulin) did not contribute to prediction of plasma CTGF-N levels. Plasma CTGF-N levels were significantly elevated in the group of diabetic patients with retinopathy compared with patients without retinopathy, but patients with retinopathy who had no signs of renal complications did not have elevated plasma CTGF-N levels. Therefore, the elevated plasma CTGF-N levels in the group of retinopathy patients are likely due to associated nephropathy rather than to the retinopathy itself. However, this does not necessarily mean that CTGF is more important in nephropathy than in retinopathy. In retinal endothelial cells and in pericytes, CTGF is induced by vascular endothelial growth factor [25]. We have observed altered CTGF distribution in retinas of diabetic patients with increased positivity of pericytes, which might relate to capillary basement membrane thickening characteristic of diabetic retinopathy (E. Kuiper, R.O.S., R.G., unpublished results). Based on the size and perfusion of the organs, however, local production of

CTGF in the kidney can be expected to contribute more to plasma levels than local production in the retina.

The observed correlation of plasma CTGF levels with markers for nephropathy raises questions as to the relevance of known determinants of the development of chronic diabetes complications to regulation of CTGF expression. It is known that CTGF and its major inducer, TGF-β, can be both induced by elevated levels of glucose and AGEs [8, 14] and that strict glycemic control is important in the prevention of development and progression of diabetes complications [4]. Therefore, a correlation might be expected between CTGF, TGF-β1, and HbA_{1c} levels in diabetic plasma. In our study, we indeed found a correlation between plasma CTGF-N and TGF-β1 ($r = 0.671$, $P <= 0.001$) and weaker correlations between CTGF and HbA_{1c} ($r = 0.355$, $P = 0.005$) and between TGF-β1 and HbA_{1c} ($r = 0.477$, $P = 0.029$). The latter is in agreement with previous observations [5] in a much larger population of diabetic subjects. Forward stepwise regression analysis, however, revealed no significant addition of HbA_{1c} to the prediction of plasma CTGF-N levels from albuminuria, creatinine clearance, and duration of diabetes. This suggests that, as is known for the development of diabetes complications, CTGF expression might be determined largely by factors other than glycemic control alone.

Given its biological activity in terms of fibrosis and matrix accumulation, it is possible that CTGF is not only a marker, but also a pathogenic factor in the development of DN and other complications. CTGF is involved in the induction of the expression of ECM components by high-glucose concentrations in renal mesangial cells and fibroblasts in vitro [12, 26]. Under diabetic conditions, TGF-β and IGF-1 are upregulated, while bone morphogenetic protein (BMP) is downregulated [2, 27]. Both inhibition of TGF-β and supplementation of BMP-7 protect against (progression of) experimental DN [28, 29]. CTGF can bind TGF-β, BMPs, and IGF-I and is also able to influence their activity. It enhances IGF-I–induced secretion of collagens I and III, and it increases the profibrotic activity of TGF-β while inhibiting BMP activity [26, 30, 31]. Thus, CTGF, induced by hyperglycemia, AGEs, or other pathways, might have an important impact on tissue remodeling and fibrosis by its contribution to the diabetes-related growth factor imbalance. Given the impressive elevation of urinary CTGF excretion in DN, it is possible that the nephropathy-associated increase of plasma CTGF reflects, at least in part, increased renal production in addition to the expected effect of reduced renal clearance [13]. In this way, plasma CTGF might be involved in nephropathy-associated aggravation of systemic (and particularly cardiovascular) complications of diabetes.

In summary, we have observed that in patients with type 1 diabetes, elevated plasma CTGF-N levels were associated with DN and correlated with level of albuminuria, impairment of creatinine clearance, and poor glycemic control. Longitudinal studies will

be needed to further investigate the possible clinical value of CTGF detection as a marker for incipient or progressive diabetes complications and, in particular, DN. To assess the possible importance of increased CTGF levels for development and progression of these disorders, animal studies will be required in which CTGF expression and activity is manipulated against a background of diabetes.

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Chapter 4

Plasma levels of connective tissue growth factor correlate with clinical markers of renal disease in type 1 diabetic patients with diabetic nephropathy

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Submitted

ABSTRACT

Plasma level and urinary excretion of the profibrotic growth factor CTGF (connective tissue growth factor/CCN-2) are increased in both human and experimental diabetic nephropathy (DN). However, the small size of previous studies has precluded analysis of the possible association of plasma CTGF level with severity of nephropathy.

Plasma CTGF levels and markers of DN were analyzed in a large cross sectional study of type 1 diabetes mellitus, involving 199 patients with DN and 188 patients with normoalbuminuria, who were matched for sex, age and duration of diabetes.

In DN patients, plasma CTGF levels were associated with both plasma creatinine ($R=0.65$, $p<0.001$) and proteinuria ($R=0.16$, $p=0.021$). Multivariate regression analysis, revealed that plasma creatinine correlated most strongly with plasma CTGF, and systolic blood pressure contributed slightly to further increase this correlation (cumulative $R=0.66$, $p<0.001$). However, in normoalbuminuric patients, age was the strongest predictor of plasma CTGF ($R=0.26$, $p<0.001$). Logistic regression analysis of the two subgroups together indicated that a standardized (1 SD) increase of plasma CTGF was associated with a twofold increased risk for DN (OR 2.0; 95%CI 1.5-2.8), which was similar to the risk associated with a 1 SD increase of HbA_{1c} (OR 2.2; 95%CI 1.6-2.9) or systolic blood pressure (OR 1.7; 95%CI 1.5-2.9).

The relatively high odds ratio of CTGF increase for DN, and the correlation of plasma CTGF with extent of renal function loss as well as proteinuria, suggest that plasma CTGF, might be both a valuable marker, and an important progression promoter of DN.

INTRODUCTION

Growth factors play an important role in the development of diabetic nephropathy (DN), a major cause of morbidity and mortality in patients with diabetes mellitus (DM). DN is characterized by proteinuria, loss of GFR, mesangial matrix accumulation, thickening of the glomerular basement membrane, and tubulointerstitial fibrosis [1]. In particular TGF-β has been implicated as a major driving force in extracellular matrix (ECM) accumulation and fibrotic processes [2]. However, its critical involvement also in controlling inflammation and as a tumor suppressor make TGF-β a less suitable target for intervention in DN. Connective tissue growth factor (CTGF, CCN2), is another profibrotic growth factor which has more recently been implicated in the development of DN [3]. Both high glucose concentrations and advanced glycation end products (AGEs) induce ECM production via CTGF [4, 5]. CTGF can act as a downstream mediator of TGF-β in ECM synthesis, but TGF-β-independent regulation of CTGF has also been reported [4, 6]. In vivo, increased CTGF expression has been shown in glomeruli of diabetic patients and animals with DN [7-11].

Previous small studies have shown that urinary CTGF excretion is elevated in both human and experimental DN [12, 13]. Recently, we showed in a large cross-sectional study of patients with type 1 DM that urinary CTGF excretion is associated with albuminuria and GFR [14]. Furthermore, we have shown that plasma CTGF levels were correlated with albuminuria and creatinine clearance in a group of well-characterized patients with type 1 DM [15]. A limitation of the latter study, however, was the relatively small group of patients with DN, which precluded analysis of a possible relation between CTGF and severity of DN.

Therefore in the present study, we assessed the clinical associations of plasma CTGF levels in a large cross sectional study of patients with type 1 DM with and without DN.

PATIENTS AND METHODS

Patients

Plasma samples were obtained from 387 well-characterized patients with type 1 DM [16]. For this, records of all adult albuminuric patients that attended the outpatient clinic at Steno Diabetes Center, Gentofte, Denmark, in 1993 who had type 1 DM and DN and had their glomerular filtration rate (GFR) measured during that year were examined. DN was diagnosed clinically if the following criteria were fulfilled: persistent UAE of $\geq$ 300 mg/24 h in at least two of three consecutive 24-h urine collections, presence of retinopathy, and no other kidney or renal tract disease. 242 Caucasian DN patients were thus identified of whom 199 (83%) accepted the invitation to participate in the study. No additional exclusion criteria were applied. In addition, 188 patients with persistent normoalbuminuria (NA), who were matched for sex, age and duration of diabetes were included in the study.

Blood samples were collected in the morning after an overnight fast. 24% of the DN patients and 88% of the NA patients received no antihypertensive treatment (AHT). The remaining patients were asked to stop their antihypertensive and diuretic treatment 8 days before examination. Of those patients, 69 (46%) DN patients and 8 (36%) NA patients refused to stop their treatment.

Urinary albumin excretion (UAE) in 24-h urine collections was determined by enzyme immunoassay [17]. Arterial blood pressure (BP) was measured twice with a Hawksley random zero sphygmomanometer in the right arm after at least 10 min of rest in the supine position. The glomerular filtration rate was measured in DN patients only, after a single intravenous injection of 3.7 MBq ^{51}Cr-EDTA at 9 am by determining the radioactivity in venous blood samples taken 180, 200, 220 and 240 min after the injection [18]. The results were standardized for 1.73 m^2 body surface area. Retinopathy was assessed by fundus photography after papillary dilatation and graded: nihil, simplex or proliferative diabetic retinopathy. Body Mass Index (BMI) was calculated as weight/height^2 (kg/m^2). Because measurement of GFR was not available in NA patients, the MDRD was calculated from plasma creatinine level, age and sex and used as estimation for the GFR in comparisons between both groups [19]. From venous blood samples, HbA_{1c} was determined by high-performance liquid chromatography (DIAMAT analyser, Bio-Rad, Richmond, CA). Plasma creatinine concentration was assessed by a kinetic Jaffé method. Smokers were defined as subjects smoking more than one cigarette, cigar or pipe per day: all others were classified as non-smokers.

The experimental design was approved by the local ethics committee, in accordance with the Declaration of Helsinki, and all patients had given their informed consent.

CTGF ELISA

Plasma concentrations of CTGF were determined by means of sandwich ELISA as described previously [15]. Both monoclonal antibodies used in this sandwich ELISA specifically bind distinct epitopes on the N-terminal half of the CTGF protein. Purified recombinant human CTGF was used for calibration (FibroGen, South San Francisco, CA). This assay detects both CTGF-N fragments as well as the full length CTGF protein. In order to avoid confusion due to differences in molecular weight of full length and different fragments of CTGF, all levels were expressed as pmol/l. The detection limit of the assay was 4 pmol/l, intra- and interassay variations were 6% and 20%, respectively.

Statistical analysis

UAE, plasma creatinine and plasma CTGF concentrations were not normally distributed and were therefore log transformed before analysis. These values are presented as medians (range); other values are given as means ± SD. A Mann-Whitney *U* test was used for comparison between groups. Frequencies were compared by χ^2-test. Forward stepwise regression analysis was used to analyze the possible correlation between plasma CTGF levels and relevant patient characteristics (age, duration of diabetes, HbA_{1c}, BMI, systolic BP (sBP), log plasma creatinine, log UAE and GFR or MDRD (as estimation of GFR)). Pearson's correlations were calculated between log plasma CTGF level and markers for DN. Logistic regression analysis was used to identify parameters that are associated with DN. The independent parameters used in this analysis were log CTGF, HbA_{1c}, duration of DM, BMI, sBP and MDRD. Odds ratios for continuous variables were standardized for one standard deviation (1 SD) difference. A p-value of <0.05 was considered statistically significant. All calculations were performed with a commercially available program (SPSS 11.5).

RESULTS

DN patients and NA patients were well matched for sex, age and duration of diabetes. Clinical data of these patients are shown in Table 1. DN patients had elevated systolic (and diastolic, data not shown) blood pressure and elevated plasma creatinine concentrations ($p<0.001$). HbA_{1c} was elevated in the DN group compared with NA patients ($p<0.001$). Patients with DN had a higher prevalence of proliferative retinopathy (69%) compared with NA patients (10%) ($p<0.001$).

Plasma CTGF levels in NA and DN patients

Plasma CTGF levels of DN patients were significantly higher than those of NA patients ($p<0.001$, Fig 1a). Patients receiving AHT and anti-diuretic treatment were asked to stop their treatment for 8 days. However, 34% of the DN patients receiving AHT refused to stop their treatment. These patients had significantly higher plasma CTGF and plasma creatinine levels, and significantly lower sBP and urinary albumin excretion ($p<0.05$) compared to DN patients who had complied with stopping their AHT (Table 1).

Table 1: Clinical characteristics of 387 patients with type 1 DM with normoalbuminuria (NA, N=188) and

	N	Sex (m/f)	Age (yrs)	Duration (yrs)	BMI (kg/m²)	HbA_{1c} (%)	Smokers (%)	AHT (%)
NA								
Total	188	114/74	42.4 ± 9.9	26.8 ± 8.5	23.6 ± 2.5	8.5 ± 1.1	42	12
DN								
Total	199	122/77	40.9 ± 9.6	27.7 ± 7.9	24.0 ± 3.3	9.6 ± 1.6*	50	76
AHT:								
No	44	28/16	37.6 ± 10.7	24.7 ± 7.1	24.2 ± 3.0	9.4 ± 1.4		
Stopped	86	57/29	41.6 ± 9.5	28.1 ± 8.4	24.6 ± 3.5	9.4 ± 1.7		
Not stopped	65	35/30	42.6 ± 8.4	29.4 ± 7.6	23.3 ± 3.1#	9.8 ± 1.5		

Some patients with previously persistent albuminuria receiving antihypertensive treatment (AHT) had urinary
Data are means ± SD or median (range). * $p < 0.001$ compared to NA, # $p< 0.05$ compared to AHT stopped.

Associations between plasma CTGF, diabetic nephropathy and clinical parameters

Within the group of DN patients, plasma CTGF level was correlated with all markers for DN, i.e. log plasma creatinine, GFR, MDRD and log UAE as well as sBP, age and duration of DM. In the NA patients, however, plasma CTGF only correlated with age (Table 2a and Fig 1c, d and e).

Forward stepwise regression analysis was performed on the data of DN and NA patients separately. In DN patients, log plasma creatinine and sBP were found to be associated with plasma CTGF level (cumulative R=0.66, p<0.001, Table 2b), while in separate analysis of NA patients, only age correlated with plasma CTGF level (R=0.26, p<0.001).

Multiple logistic regression analysis with nephropathy as dependent variable was performed to investigate the contribution of plasma CTGF level to risk of DN. After adjustment for duration of diabetes, BMI, sBP, HbA_{1c} and MDRD, a standardized (1 SD) increase of log plasma CTGF resulted in a 2.0-fold increase of the chance of having DN (OR 2.0, 95% CI 1.5-2.8). Also a 1 SD elevation of HbA_{1c} (OR 2.2; 95% CI 1.6-2.9) and of sBP (OR 1.7; 95% CI 1.5-2.9) were associated with DN. In addition, a decrease in MDRD with 1 SD resulted in a 2-fold increased risk of having DN (OR 0.5; 95% CI 0.32-0.62).

diabetic nephropathy (DN, N=199).

plasma creat (µM)	GFR (ml/min/ 1.73m²)	MDRD (ml/min/ 1.73m²)	sBP (mm Hg)	Retinopathy (nihil/simplex/ proliferative)	UAE (mg/24 h)
76 (40-116)	---	91 ± 27	132 ± 18	67/103/19	8 (1-30)
103 (54-684)*	74 ± 34	63 ± 30*	151 ± 23*	0/62/137*	796 (16-14545)
80 (56-166)	102 ± 26	81 ± 26	135 ± 14	0/20/24	636 (96-7252)
104 (54-587)	70 ± 30	61 ± 27	161 ± 21	0/23/63	1311 (35-14545)
117 (57-684)#	60 ± 32	55 ± 32	144 ± 22#	0/18/47	609 (16-5808)#

albumin excretion (UAE) < 300 mg/24 h. Patients with AHT were asked to stop their treatment for 8 days.

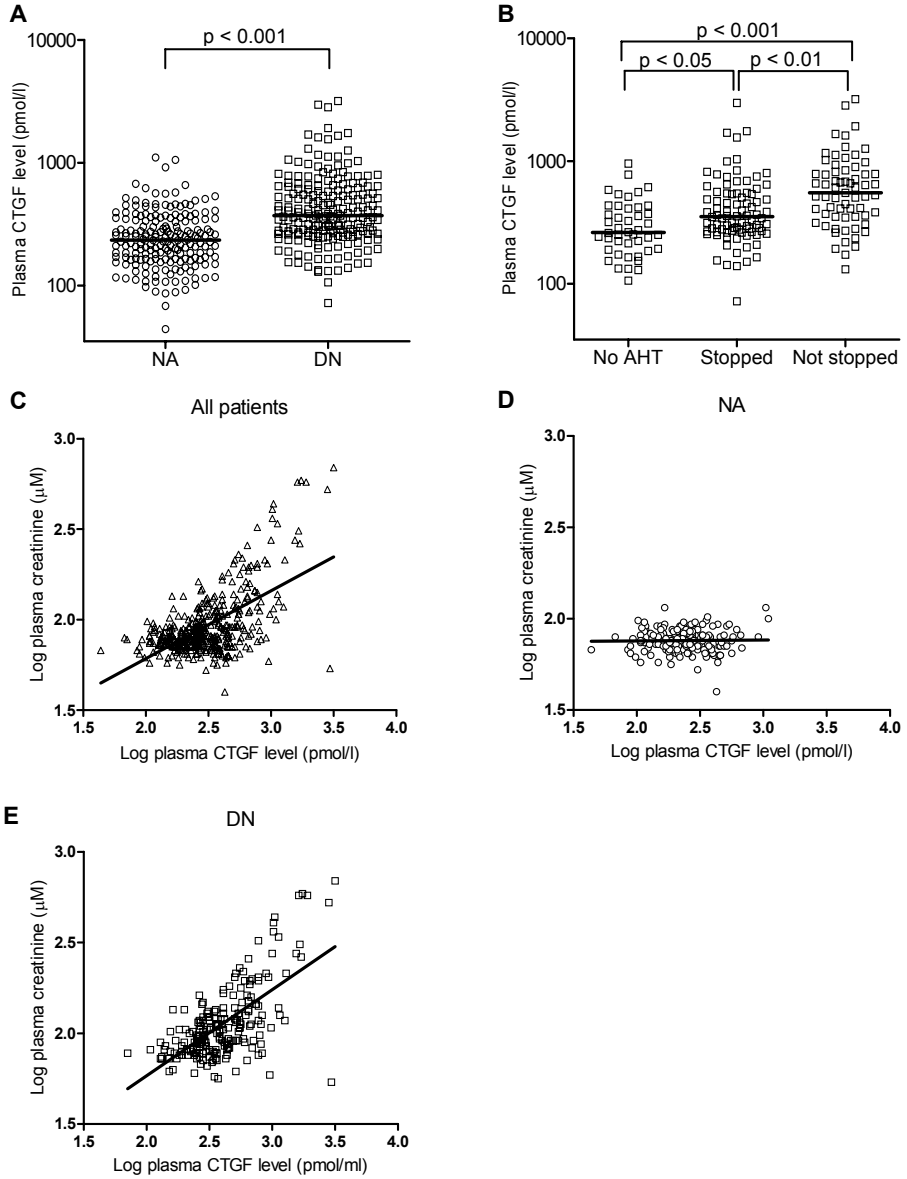

Figure 1:. Plasma CTGF level and its correlation with plasma creatinine in different patient groups. A: Plasma CTGF level in normoalbuminuric diabetic patients (NA) and diabetic patients with nephropathy (DN). NA: 233 (44-1103) pmol/l, and DN: 371 (72-3191) pmol/l, (median (range)), p<0.001. B: Plasma CTGF levels in patients with nephropathy without and with antihypertensive treatment (AHT), who did or did not stop their medication. No AHT: 261 (106-952), Stopped: 352 (72-2970) and Not stopped: 549 (131-3191) pmol/l. No AHT vs. Stopped, No AHT vs. Not stopped, and Stopped vs. Not stopped: p<0.05, p<0.001, and p<0.01 respectively. C, D and E**:** Correlation of plasma CTGF level with plasma creatinine in different groups of diabetic patients. C: all patients: R=0.597, p<0.001, D: normoalbuminuric patients: R=0.018, p=0.807, and E: patients with nephropathy: R=0.646, p<0.001.

DISCUSSION

This is the first large study of CTGF levels in plasma samples of patients with type 1 diabetes. Our results confirm and extend previous observations in a much smaller "pilot" study [15], which had indicated already that plasma CTGF levels are significantly elevated in DN patients as compared to NA patients. To this we add that, within a large population of type 1 diabetic patients with DN, plasma CTGF levels correlate most strongly with severity of renal disease.

In DN patients, plasma CTGF levels correlated most strongly with plasma creatinine, but also with GFR and UAE. In addition, significant correlations were observed between plasma CTGF, sBP and age of DN patients. A large variation in plasma CTGF levels was observed within the DN as well as the NA group. In DN patients, plasma CTGF levels strongly correlated with markers for DN. In NA patients, however, plasma CTGF level only correlated with age, despite significant variation in estimated GFR (MDRD) and even more in plasma CTGF level. Plasma CTGF in the NA group showed considerable overlap with that in the DN group, but since the mean duration of diabetes is 27 years in both patient groups, it is unlikely that a significant number of these NA patients would still develop DN in the future. Therefore an association of plasma CTGF level with susceptibility for DN in the NA group can almost be excluded. At present, it remains unclear which factors determine the variation in plasma CTGF levels among NA patients, but apparently these do not simply reflect differences in renal clearance.

Forward stepwise regression analysis was used to identify parameters that might predict plasma CTGF level in DN patients. In the DN patients, plasma creatinine was found to be the main predictor of plasma CTGF level (R=0.65). Although also albuminuria did correlate (weakly) with CTGF (R=0.16), addition of this parameter did not significantly improve prediction of plasma CTGF by plasma creatinine. In NA patients no correlation was found between plasma CTGF levels and GFR or low-grade albuminuria.

High blood pressure is an important risk factor for the development and progression of DN in diabetic patients [20] and in vitro studies have revealed that CTGF expression is stimulated by shear stress as well as static pressure [21]. Systolic BP was significantly associated with plasma CTGF level, but addition of sBP to plasma creatinine improved this prediction only marginally (cumulative R=0.66 vs. R=0.65 for creatinine alone). This suggests that in vivo contribution of these factors to plasma levels of CTGF may be significant but is at most relatively limited.

Of note, logistic regression analysis revealed that association of plasma CTGF with DN was of similar strength as the association of HbA_{1c} and sBP with DN. In a recent large cross-sectional study of patients with type 1 DM [14] we observed that also urinary

Table 2: Correlations of plasma CTGF

A: Pearson's correlations of log plasma CTGF level in DM patients with NA and DN

Parameter	NA patients	DN patients
Log plasma creatinine	R= 0.018, p=0.807	**R=0.646, p<0.001**
GFR	---	**R=-0.603, p<0.001**
MDRD	R= 0.052, p=0.481	**R=-0.464, p<0.001**
Log UAE	R= 0.031, p=0.668	**R= 0.164, p=0.021**
sBP	R= 0.126, p=0.085	**R= 0.399, p<0.001**
Age	**R= 0.260, p<0.001**	**R= 0.242, p<0.001**
Duration	R= 0.107, p=0.144	**R= 0.181, p=0.010**

B: Forward stepwise regression analysis:

Log plasma CTGF level can be predicted from a linear combination of the following variables:

Parameter	Cumulative R	p-value
NA patients:		
Age	R=0.260	p<0.001
DN patients:		
Log plasma creatinine	R=0.646	p<0.001
Systolic blood pressure	R=0.662	p=0.008

Not normally distributed parameters (i.e. plasma CTGF, plasma creatinine and UAE) were log transformed to obtain a normal distribution of the data.

CTGF excretion is associated with albuminuria and GFR. Logistic regression analysis showed that the association of urinary CTGF excretion with DN is equally strong as the association of HbA_{1c} and sBP with DN. Of interest, this odds ratio of urinary CTGF excretion for presence of DN (OR= 2.3) was of similar magnitude as the one we observed for plasma CTGF (OR = 2.0), and also odds ratios of HbA_{1c} and sBP for DM were almost identical in both studies. This might suggest an association between plasma and urinary CTGF levels. However, because in previous investigations as well as in the current study, urine and plasma samples have not been available from the same patients, the relation between plasma- and urinary CTGF still remains unclear.

Plasma CTGF levels are determined by production and clearance of CTGF. Expected determinants of clearance of CTGF are GFR and proteinuria. Unfortunately, pharmacokinetic characteristics of CTGF are not known. Since at least a proportion of CTGF (MW 36-38 kDa) and its fragments (approximately 20 kDa and smaller [22])

would be expected to pass the glomerular filter, plasma CTGF levels might depend at least in part on renal function (GFR). If CTGF would pass freely through the GBM, assuming equal CTGF production, an inverse correlation of plasma CTGF with GFR would be expected in both NA and DN patients. However, this correlation appears to exist only in DN and not in NA patients, which might suggest that CTGF is filtered by proteinuric glomeruli only, and that in NA patients it can be retained in the circulation, for example due to yet unknown physical association of CTGF with other proteins.

As far as CTGF production is concerned, previous studies have shown that CTGF protein as well as renal mRNA expression are strongly increased in DN patients and experimental animal models of DN [8, 10]. Because 20% of the systemic blood flow passes through the kidneys, it seems not unlikely that excess CTGF synthesis in the diseased kidney would contribute to increased plasma CTGF level. As a consequence, the higher plasma CTGF levels in DN patients might, at least partly, be caused by increased renal CTGF synthesis, which is also suggested by the fact that both plasma CTGF level and urinary CTGF excretion are increased in DN patients [12, 13]. However, the large overlap between plasma CTGF values of NA and DN patients indicates that also extra-renal factors might have important influence on plasma CTGF level. This is also suggested by our recent observation that albuminuria is the best predictor of urinary CTGF excretion in DM, while in contrast; plasma CTGF levels are not predicted by albuminuria after correction for (estimated) GFR.

Since, *in vitro* CTGF expression is induced by high glucose and AGEs [3], the metabolic condition (reflected by HbA_{1c}) would be expected to be a major determinant also of *in vivo* CTGF production [4, 5]. However, despite significantly higher levels of both HbA_{1c} and plasma CTGF in DN patients, we found no correlation between these parameters, neither in DN patients nor in NA patients.

In addition to the metabolic condition also vasculopathy related to sBP and retinopathy [23, 24] might be involved in regulation of CTGF production. Systolic BP correlated with plasma CTGF levels only in DN patients and altered local CTGF expression has been noted in the retina of diabetic patients [25]. The observed increase of CTGF in pericytes was suggested to relate to capillary basement membrane thickening, a characteristic not only of diabetic retinopathy [25], but an almost ubiquitous phenomenon in complications of DM [26]. Based on size and perfusion of the organs, increased local production of CTGF in the kidney would be expected to contribute more to plasma levels than increased local production in the retina, but on the other hand, signs of retinopathy might serve as a structural marker of systemic microvascular damage [27], in which case a stronger impact on plasma CTGF level would be anticipated. However, plasma CTGF levels in patients with and without retinopathy were not significantly different, neither in the NA group, nor in DN patients. Thus, if retinopathy is indeed a

marker for systemic microvascular disease, it seems that the latter does not significantly affect plasma CTGF level.

It has been reported that renal CTGF expression is increased by Angiotensin II [28], and urinary CTGF excretion in DN is reduced by blocking the renin-angiotensin system [12, 29]. However, in the present study, DN patients who did stop their AHT had lower plasma CTGF levels than those who did not. DN patients who stopped their AHT also had lower plasma creatinine levels, while UAE and sBP were higher, as expected [30]. These observed lower plasma CTGF and plasma creatinine levels might partly result from GFR-increase, as GFR tended to be, although not significantly, higher in the patients off-AHT ($p=0.06$). We are not informed about the motives of patients who refused to stop taking AHT. It cannot be excluded that these might relate to more severe or advanced disease as compared to patients who did stop their AHT, but from our current knowledge we don't have a satisfactory explanation for their higher plasma CTGF.

The considerable scatter and overlap between NA and DN patients, and the moderate strength of the associations of plasma CTGF with markers for DN, might suggest that the value of plasma CTGF as a marker for DN severity in cross-sectional survey is relatively limited. However, it is important to notice that standardised, 1 SD increase of plasma CTGF was associated with a similar odds ratio for having DN as a 1 SD increase of HbA_{1c} or sBP, the two major and most firmly established risk factors for DN [20]. The same has been observed with respect to urinary CTGF [14]. Therefore, we suggest that also (plasma) CTGF might be an important progression promoter of DN and a target for future therapeutic and preventive intervention.

From this first large study of plasma CTGF in patients with type 1 diabetes mellitus we conclude that plasma CTGF levels are correlated with presence and severity of diabetic nephropathy. Longitudinal studies will be needed to elucidate the possible importance of plasma- and urinary CTGF as a prognostic factor in development and progression of DN.

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Chapter 5

Temporal expression profile and distribution pattern indicate a role of connective tissue growth factor in diabetic nephropathy in mice

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ABSTRACT

Connective tissue growth factor (CTGF) is overexpressed in diabetic nephropathy (DN) and has therefore been implicated in its pathogenesis. The objective of the present study was to determine the tissue distribution of increased CTGF expression and the relationship of plasma, urinary, and renal CTGF levels to the development and severity of DN. We studied the relationship between CTGF and renal pathology in streptozotocin (STZ)-induced diabetes in C57BL/6J mice. Diabetic and age-matched control mice were killed after 1, 2, 4, and 9 wk of diabetes. In addition, key parameters of diabetes and DN were analyzed in 10-mo-old diabetic ob/ob mice and their ob/+ littermates. STZ-induced diabetic mice showed a significantly increased urinary albumin excretion after 1 wk and increased mesangial matrix score after 2 wk. Increased renal fibronectin, fibronectin ED-A, and collagen IVα1 expression, as well as elevated plasma creatinine levels, were observed after 9 wk. After 2 wk, CTGF mRNA was upregulated threefold in the renal cortex. By 9 wk, CTGF mRNA was also increased in the heart and liver. In contrast, transforming growth factor-β1 mRNA content was significantly increased only in the kidney by 9 wk. Renal CTGF expression was mainly localized in podocytes and parietal glomerular epithelial cells, and less prominent in mesangial cells. In addition, plasma CTGF levels and urinary CTGF excretion were increased in diabetic mice. Moreover, albuminuria strongly correlated with urinary CTGF excretion ($R = 0.83$, $P < 0.0001$). Increased CTGF expression was also demonstrated in type 2 diabetic ob/ob mice, which points to a causal relationship between diabetes and CTGF and thus argues against a role of STZ in this process. The observed relationship of podocyte- and urinary CTGF to markers of DN suggests a pathogenic role of CTGF in the development of DN.

INTRODUCTION

Diabetic nephropathy (DN) is a major complication of diabetes mellitus (DM) and an important cause of morbidity and mortality in diabetic patients. Early characteristics of DN are albuminuria, renal and glomerular hypertrophy, extracellular matrix (ECM) accumulation, and thickening of the glomerular basement membrane (GBM) [1]. Growth factors play an important role in the development of diabetic complications [2-5]. One of the major growth factors involved in ECM accumulation in many fibrotic disorders, including DN, is transforming growth factor-β (TGF-β) [2]. Inhibition of TGF-β results in prevention of fibrosis under diabetic conditions [6]. However, TGF-β has also important anti-proliferative and anti-inflammatory effects, which makes it a less suitable target for therapeutic intervention of DN.

Connective tissue growth factor (CTGF), also known as CCN-2 [7], is a profibrotic growth factor critically involved in TGF-β-induced [4] but also in TGF-β-independent fibrotic processes [8, 9]. In renal cells in vitro, CTGF can be induced by TGF-β, mechanical strain, and diabetic conditions, such as high levels of glucose and AGEs [1, 4, 10-13]. In vivo, overexpression of CTGF has been reported in renal tissue of both diabetic patients and animals with DN [14-16]. Aminoguanidine, an inhibitor of advanced glycation end product formation, reduced CTGF and fibronectin overexpression in streptozotocin (STZ)-induced diabetic rats [17].

Upregulation of growth factors in several glomerular cells has been implicated in DN. For example, increased TGF-β and PDGF-BB expression in diabetic glomeruli seems mainly localized to the mesangial cells [18-20], but increased TGF-β expression has also been reported for glomerular endothelial cells [21]. VEGF overexpression, on the other hand, is found mainly in podocytes [20, 22]. In DN, the main site of renal CTGF overexpression is the glomerulus [14-16], but it is not known which cells within the glomerulus contribute most importantly to this. Cell-specific variation in glomerular expression and activity of growth factors has important implications for the pathogenic role of these factors. Understanding the kinetics of renal CTGF expression and the magnitude of increased CTGF levels in plasma and urine is important for interpreting the source of CTGF in these different compartments and for targeting therapeutic intervention strategies.

Urinary CTGF excretion is increased in STZ-induced diabetic rats as well as in patients with DN [23, 24]. In addition, plasma CTGF levels are increased in patients with DN and correlate with both HbA_{1c} and other parameters of DN [25]. Thus far, however, no integrative analysis has been performed on CTGF expression levels in plasma, urine, and renal tissue in relation to the development of DN. Therefore, we set out to explore the cellular origin of CTGF and its relationship to plasma, urinary, and renal CTGF levels in time, in association with development and severity of DN.

METHODS

Animals

Two models of diabetes were studied. In the first model, diabetes was induced in female C57BL/6J mice (12 wk old, Harlan, Horst, The Netherlands) by a single intraperitoneal injection of STZ (200 mg/kg, Sigma, St. Louis, MO) dissolved in 10 mM sodium citrate buffer (pH 4.6). Control animals were injected with sodium citrate buffer alone. All animals were housed in a room with constant temperature, on a 12:12-h light-dark cycle and fed a standard pellet laboratory chow and water ad libitum. Presence of diabetes was determined 3 days after STZ injection by measurement of blood glucose levels (Medisense Precision Xtra apparatus, Abbott, Bedford, IL). When necessary, as indicated by a decrease in body weight of >10% within 2 days together with blood glucose levels >20 mmol/l, slow-release insulin pellets (Linshin Canada, Ontario, Quebec) were implanted subcutaneously. Every other week and before death, STZ-diabetic (STZ-DM) mice were kept in metabolic cages for 4 h and urine was collected and stored at –70°C. Mice were killed after 1, 2, 4, and 9 wk of DM. Control animals were killed 2 and 9 wk after citrate injection.

In the second model, genetically determined leptin deficiency in homozygous ob/ob mice causes insulin resistance and hyperglycemia already at a young age [26]. Transgenic mice expressing human amyloid polypeptide in their pancreatic islets develop islet amyloid, particularly male animals when crossed back with ob/ob mice (22). Female hIAPP-transgenic ob/ob mice (n = 5, C57BL/6J background) and their non-diabetic hIAPP-transgenic ob/+ littermates (n = 6) were studied when 10 mo old. Urine was collected in metabolic cages before death.

In both models, blood was collected in EDTA, and plasma was stored at –70°C. Body and organ weights were determined. Tissues were frozen in liquid nitrogen or fixed in buffered formalin (10%) and then embedded in paraffin for light microscopy. The experiments were performed with the approval of the Animal Ethical Commission of the University of Utrecht.

Analysis of plasma and urine

Plasma fructosamine levels were measured using a commercially available test (F. Hoffmann-La Roche, Basel, Switzerland). Albumin levels in urine were determined by means of sandwich ELISA using a goat anti-mouse albumin antibody (Bethyl Laboratories, Montgomery, TX). Creatinine concentrations in plasma and urine were determined using an enzymatic assay (J2L Elitech, Labarthe Inard, France).

Table 1: Primer sequences used in quantitative RT-PCR

CTGF
Forward: 5'-CACAGAGTGGAGCGCCTGTTC-3'
Reverse: 5'-GATGCACTTTTTGCCCTTCTTAATG-3'
TGF-β1 [27]
Forward: 5'-GCAACATGTGGAACTCTACCAGA-3'
Reverse: 5'-GACGTCAAAAGACAGCCACTCA-3'
Fibronectin [27]
Forward: 5'-CAGGAGCCAAGAGTGAAGAAC-3'
Reverse: 5'-GGAAATAATTCTGGCTCATAGCTACT-3'
Fibronectin ED-A
Forward: 5'-GCAGTGACCAACATTGATCGC-3'
Reverse: 5'-ACCCTGTACCTGGAAACTTGCC-3'
Collagen IVα1
Forward: 5'-ATTCCTTCGTGATGCACACC-3'
Reverse: 5'-GTGGGCTTCTTGACATCTC-3'
TBP
Forward: 5'-CAGGAGCCAAGAGTGAAGAAC-3'
Reverse: 5'-GGAAATAATTCTGGCTCATAGCTACT-3

CTGF, connective tissue growth factor; TGF, transforming growth factor; TBP, TATA box-binding protein.

Mesangial matrix score

The amount of mesangial matrix was estimated in periodic acid-Schiff-stained sections from all animals. In each slide, 25 glomeruli were scored with a range from 1 to 4 by 2 persons, both blinded to the origin of the slide. The mean of both scores was used as the mesangial matrix score (MMS).

Quantitative RT-PCR

The mRNA levels of CTGF, TGF-β1, and several ECM components in total renal cortex were quantified by real-time quantitative PCR. For this purpose, RNA was isolated from frozen sections of renal cortex using a RNeasy minikit (Qiagen, Hilden, Germany). cDNA synthesis was performed on 3 µg of RNA using oligo-dT12–18 and Superscript RT (Invitrogen, Carlsbad, CA).

Quantitative PCR was performed using a SYBR Green kit (Applied Biosystems, Foster City, CA) and analyzed on an ABI PRISM 7700 Sequence Detector System (Applied Biosystems). Genes and primer sequences are listed in Table 1. The thermal cycling comprised a denaturation step at 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. To examine whether only one single PCR product was generated, the PCR products were analyzed using a heat dissociation protocol. Quantitative values were calculated from the threshold PCR cycle numbers, which were

derived from the exponential phase of each PCR reaction. The relative mRNA level in each sample was normalized for TATA box-binding protein mRNA content [28].

CTGF ELISA of plasma and urine samples

CTGF levels in plasma and urine were determined by means of a sandwich ELISA using an affinity-purified polyclonal antibody and a monoclonal antibody. These two antibodies react with distinct epitopes in the NH_2-terminal half of the CTGF protein (CTGF-N; FibroGen, South San Francisco, CA).

Microtiter plates were coated overnight at 4°C with 50 µl of capture goat-anti-CTGF-N polyclonal antibody (10 µg/ml) in coating buffer (0.05 M sodium bicarbonate, pH 9.6). Subsequently, the plates were blocked with 1% BSA in PBS for 2 h at room temperature and washed with wash buffer (PBS/0.05% Tween 20). Plasma samples were diluted 1:10 and urine samples 1:2 in assay buffer (50 mM Tris, pH 8.0, 0.1% BSA, 50 mg/l sodium heparin, 0.1% Triton X-100) and 100 µl of diluted sample were added to each well. Purified recombinant human CTGF (FibroGen) was used as a standard. Plates were incubated for 2 h at room temperature, washed, and incubated with 100 µl (4 µg/ml in assay buffer) monoclonal human anti-CTGF-N antibody produced by transgenic mice generating human antibodies (FibroGen). Plates were washed and incubated with 100 µl/well goat anti-human IgG alkaline phosphatase (diluted 1:2,000 in assay buffer, Sigma). Plates were washed again, and 100 µl of substrate solution containing p-nitrophenylphosphate (Sigma) were added to each well. After a 20- to 30-min incubation in the dark, absorbance was read at 405 nm on a Bio-Rad microplate reader. As this ELISA cannot discriminate between full-length and different NH_2-terminal fragments of CTGF, all levels are expressed as picomoles per liter.

CTGF immunohistochemistry

Four-micrometer sections were cut from formalin-fixed and paraffin-embedded tissue samples and mounted on silan-coated slides. Slides were fixed overnight at 56°C in an incubator. Sections were deparaffinized and rehydrated. A 3-min incubation of the slides at 37°C with protease XXIV (4.4 U/ml phosphate, Sigma) was performed for epitope retrieval. After 2 min of washing with distilled water, slides were treated for 20 min with a 1.5% hydrogen peroxide solution in PBS to block endogenous peroxidase activity. Tissue sections were washed three times for 3 min in PBS-Tween and incubated for 1 h with 8 µg/ml CTGF-specific human monoclonal antibody (FibroGen) in PBS/1% BSA. Sections were rinsed three times for 3 min in PBS-Tween and incubated for 30 min with rabbit-α-human IgG (DakoCytomation, Copenhagen, Denmark) diluted 1:1,500 in PBS/1% BSA containing 5% normal mouse serum (DakoCytomation). After three times 3 min of washing with PBS-Tween, sections were incubated with goat α-rabbit Powervision-PO (Klinipath, Duiven, The Netherlands) for 30 min followed by three

times 3 min of washing with PBS-Tween. Subsequently, the sections were developed with Nova RED (Vector Laboratories, Burlingame, CA) and rinsed with distilled water. Nuclear staining was performed with hematoxylin and developed in running tap water for 10 min. Tissue sections were dehydrated and covered with Pertex and a coverslip.

CTGF in situ hybridization

A 542-bp cDNA fragment of rat CTGF (GenBank GI no. 5070343 496–1037) was amplified by PCR using the following sense and antisense primers: 5'-ATTTAGGTGACACTATAGAAGAGGCGTGTGCACTGCCAAAGAT-3' and 5'-TAATACGACTCACT ATAGGGAGAGCAGCCAGAAAGCTCAAACTTGA-3', respectively. In vitro transcription from the cloned amplicon as a template was performed using SP6 or T7 RNA polymerases and digoxigenin (DIG)-conjugated UTP (Roche) to produce DIG-labeled sense or antisense riboprobes.

Deparaffinized sections of formalin-fixed tissue were preincubated with proteinase K (Invitrogen) and hybridization buffer and subsequently with 250 ng/ml CTGF DIG-labeled riboprobe in hybridization buffer [50% formamide, 5x SSC, 1% blocking reagent (Roche), 5 mM EDTA, 0.1% Tween 20, 0.1% CHAPS, 0.1 mg/ml heparin, 1 mg/ml yeast tRNA] at 70°C for 16 h. After washing, blocking of nonspecific binding sites by 2% blocking reagent and 10% normal goat serum in PBS/T, sections were incubated with alkaline phosphatase-conjugated sheep anti-DIG antibodies (Roche). After washing, alkaline phosphatase activity was detected with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (Roche). Subsequently, immunohistochemistry was performed for collagen type IV to provide a counterstain that does not interfere with the cellularly localized in situ hybridization signals. Endogenous peroxidase activity was inactivated, nonspecific protein binding sites were blocked with 10% normal goat serum in PBS, and sections were incubated with rabbit anti-collagen type IV (Euro-Diagnostica, Arnhem, The Netherlands) and, after washing, with poly-horseradish peroxidase goat anti-rabbit IgG (Powervision, Immunovision Technologies, Brisbane, CA). Horseradish peroxidase activity was detected using 3,3'-diaminobenzidine and hydrogen peroxide. Sections were dehydrated and mounted with Pertex.

Statistics

Data are expressed as means ± SE. Differences between the control group and diabetic groups were determined by Mann-Whitney U-test or Kruskal-Wallis analysis followed by a Dunn's test where appropriate. Pearson's correlations were used. Skewed data sets were log-transformed. A value of $P < 0.05$ was considered to be significant.

RESULTS

General characteristics of mouse models of diabetes

After injection with STZ, ~75% of the mice developed hyperglycemia with blood glucose levels exceeding 15 mmol/l within 3 days. Mice that did not develop hyperglycemia within 1 wk were excluded from the study. General characteristics of STZ-DM and ob/ob, as well as of appropriate nondiabetic control mice, are presented in Tables 2 and 3. Mean blood glucose levels of STZ-DM mice exceeded the upper limit of quantification (27.8 mmol/l) 1 wk after development of diabetes and remained elevated throughout the study. Blood glucose levels in control mice ranged from 4 to 8 mmol/l. Insulin implants, providing a constant release of insulin, stabilized the condition of the STZ-DM mice, although blood glucose levels in these animals showed a high variation during the day. Also, in ob/ob mice blood glucose levels at 10 mo of age were increased above the upper limit of quantification (27.8 mmol/l).

The plasma fructosamine level, an indicator of metabolic control [29], was significantly higher in STZ-DM and ob/ob mice compared with their respective controls. In 9-wk STZ-DM mice, all receiving insulin, plasma fructosamine levels were lower compared with earlier time points but still significantly elevated compared with those in control mice.

Table 2: General characteristics of STZ-DM and control mice

		STZ-DM			
		Duration of DM (weeks)			
	Control	1	2	4	9
n	14	6	5	5	6
Blood glucose (mmol/l)	6.8±0.4	> 27.8	> 27.8	> 27.8	Variable
Plasma fructosamine (µmol/l)	183±3	329±7*	314±25*	366±14*	275±13*
Body wt *day 0* (g)	21.5±0.2	19.9±0.1	20.4±0.3	22.1±0.2	22.1±0.1
Body wt at death (g)	24.2±0.3	16.4±0.4*	17.8±0.4*	19.3±0.6*	21.5±0.2*
Body wt/body wt *day 0* (%)	113±2	83±2*	87±2*	87±3*	97±1*
Kidney wt (mg)	136±2	150±7	167±4*	163±9*	148±6
Kidney wt/body wt (%)	1.12±0.02	1.82±0.08*	1.88±0.07*	1.69±0.11*	1.38±0.06
Plasma creatinine (mg/dl)	0.18±0.02	0.36±0.05*	0.35±0.03*	0.30±0.05	0.26±0.02*
Albuminuria (mg/g creatinine)	27±6	731±229*	223±97	968±248*	113±35*

Values are means ± SE. *n*, No. of mice; STZ-DM, streptozotocin-induced diabetes mellitus.

* $P < 0.5$ compared with non-diabetic control.

STZ-DM mice lost weight during the experiment, whereas control mice gained weight due to normal growth. After an initial drop of ~10%, body weight of the STZ-DM mice receiving insulin pellets remained almost constant throughout the study. As no differences in key parameters were observed between 2- and 9-wk control mice, these mice served as one control group; ob/ob mice became progressively obese but remained clinically stable without insulin supplementation.

Table 3: General characteristics of non-diabetic ob/+ and diabetic ob/ob mice

	ob/+	ob/ob	p-value
n	5	6	
Blood glucose (mmol/l)	9.7±0.6	>27.8	0.004
Plasma fructosamine (µmol/l)	180±2	319±13	0.004
Body wt (g)	41.3±1.0	49.1±2.9	0.052
Kidney wt (mg)	175±5	208±6	0.009
Kidney wt/body wt (%)	0.42±0.02	0.42±0.02	0.792
Plasma creatinine (mg/dl)	0.29±0.08	0.43±0.04	0.106
Albuminuria (mg/g creatinine)	69±10	653±200	0.004
Plasma CTGF (pmol/l)	485±44	4320±1607	0.126
Urinary CTGF (pmol/g creatinine)	36±6	9099±2283	0.004

Values are means ± SE. *n*, No. of mice.

Development of nephropathy

Already after 1 wk of STZ-DM, a significant increase in plasma creatinine was observed, which persisted at 2, 4, and 9 wk. Especially at the earliest time points, the rise in plasma creatinine might have been caused, at least in part, by dehydration. Because we have no data on actual creatinine clearance, it remains unclear whether the elevated plasma creatinine levels at later time points reflect loss of renal function. STZ-DM mice showed an increase in albuminuria as early as 1 wk after development of DM, and albuminuria persisted during the experiment. However, large variations in albuminuria levels were observed among individual STZ-DM mice (Fig. 1, A and B). Kidney mass was significantly increased after 2 and 4 wk of STZ-DM and had subsequently decreased at 9 wk (Table 2), indicating a fast but transient development of renal hypertrophy after induction of DM. In contrast, the mesangial matrix expanded progressively over time, also from 2 wk onward in STZ-DM mice (Fig. 1, C and D).

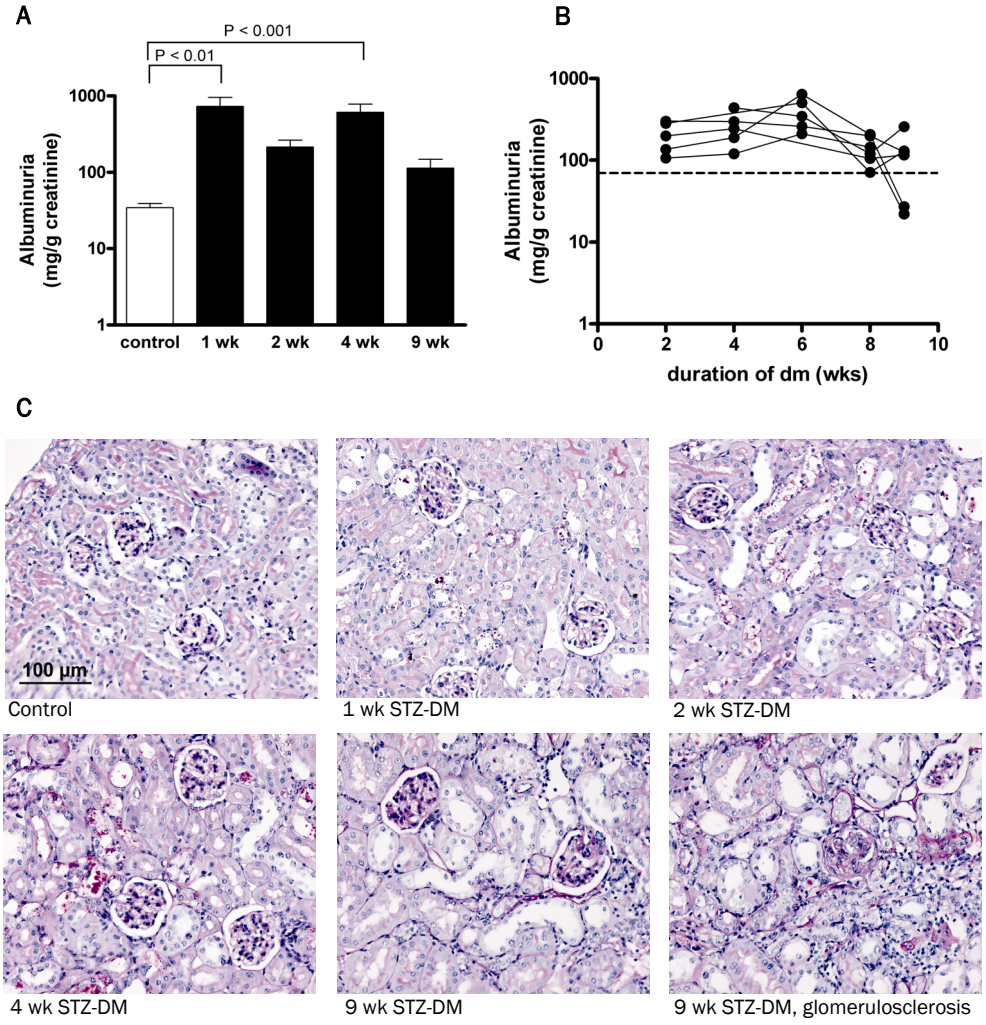

Figure 1: Development of diabetic nephropathy in streptozotocin-induced diabetic (STZ-DM) mice. A: albuminuria in control mice and diabetic mice 1, 2, 4, and 9 wk after development of diabetes mellitus (DM). B: course of albuminuria in diabetic mice. The dashed line represents mean ± 2 SD of control. C: periodic acid-Schiff-stained kidney sections of control and diabetic mice 1, 2, 4, and 9 wk after development of DM showing increasing kidney damage in diabetic animals. All pictures are taken at similar magnification. D: mesangial matrix score as measure for glomerular ECM accumulation of control and diabetic mice.

Besides glomerular alterations, tubulointerstitial damage was observed, as exemplified by epithelial simplification and degeneration and the presence of protein casts and cellular infiltrate (Fig. 1C).

Because hyperglycemia may lead to upregulation of specific ECM proteins [30], quantitative RT-PCR was performed for total fibronectin, its fibrosis-associated splice variant fibronectin ED-A, and collagen IVα1 on total renal cortex RNA of control mice and of diabetic mice. After 2 and 4 wk of STZ-DM, renal mRNA expression of all these ECM genes tended to increase, which became significant at 9 wk of STZ-DM (Fig. 2).

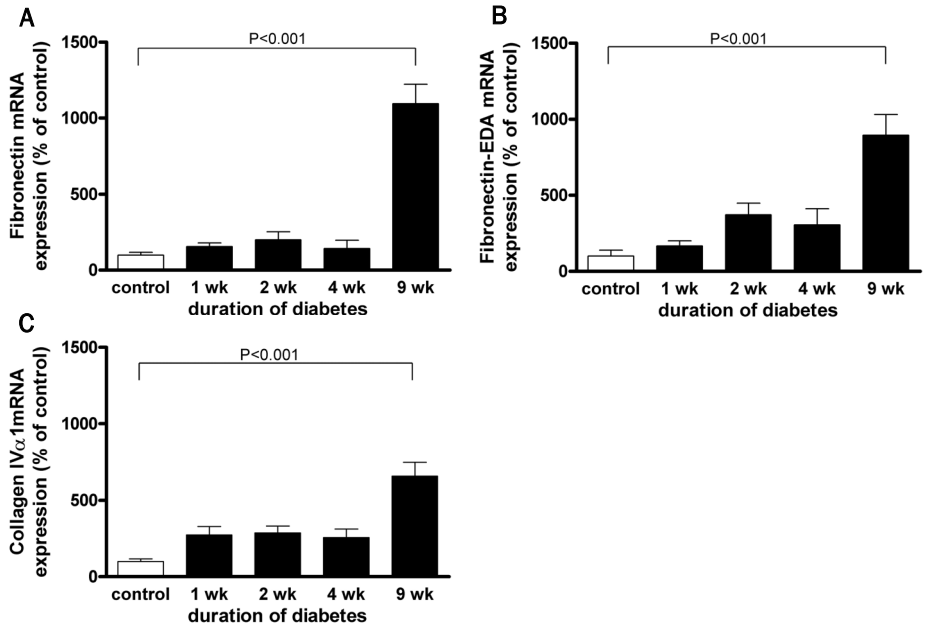

Figure 2: Renal mRNA expression levels of ECM proteins in STZ-DM mice. A: fibronectin. B: fibronectin ED-A. C: collagen IVα1. Data are corrected for the mRNA expression of the housekeeping gene encoding TATA box-binding protein and are represented as percentage relative to controls (means ± SE).

Together, these functional and structural data confirm development of nephropathy in our mouse model of STZ-induced diabetes mellitus.

At 10 mo of age ob/ob mice also showed albuminuria, but no significant increase in plasma creatinine and relative kidney weight was observed compared with the non-diabetic ob/+ littermates (Table 3).

Increased CTGF expression in diabetic animals

Plasma CTGF levels were significantly increased in 9-wk STZ-DM animals compared with controls ($P < 0.0001$, Fig. 3A). Urinary CTGF levels of most control mice were below the detection limit of the sandwich ELISA (<50 pmol/l). However, in STZ-DM mice, urinary CTGF levels were readily detectable. After correction for urinary creatinine excretion, urinary CTGF levels appeared significantly increased at all time points (Fig.

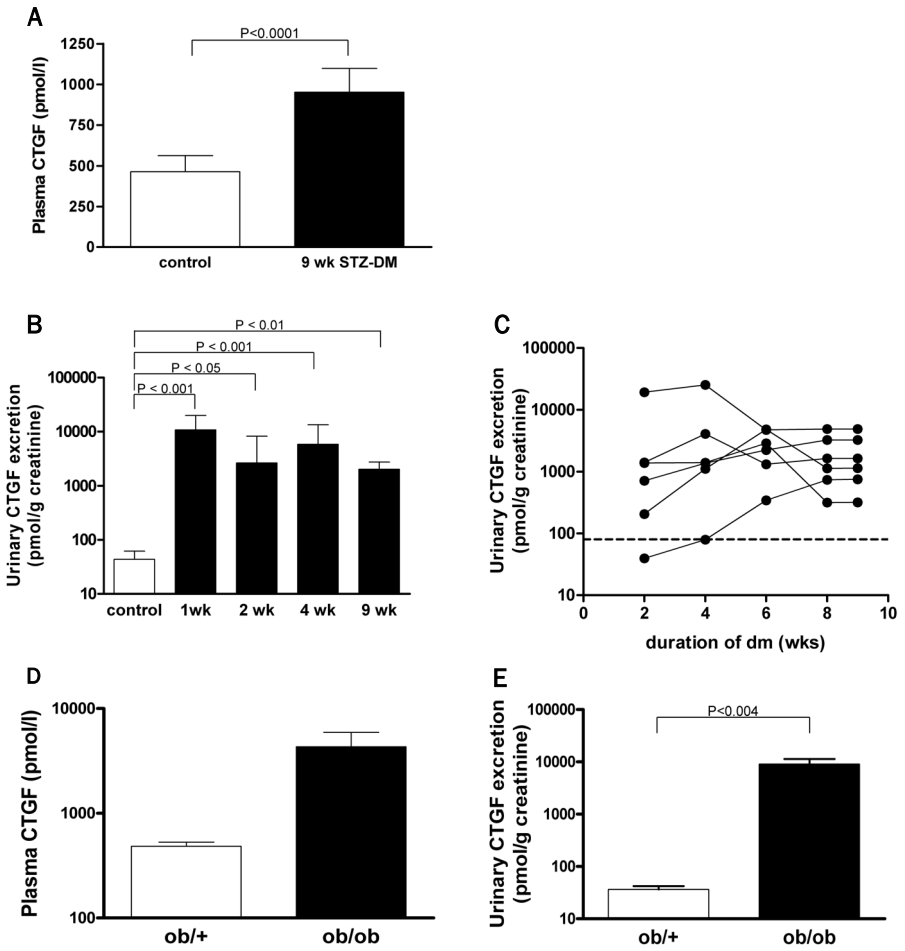

Figure 3: Connective tissue growth factor (CTGF) levels in plasma and urine of STZ-DM and control animals. A: plasma CTGF levels in control and 9-wk STZ-DM mice. B: urinary CTGF excretion of control and 1-, 2-, 4-, and 9-wk STZ-DM mice. C: course of urinary CTGF excretion at different time points in individual STZ-DM mice. The dashed line represents mean ± 2 SD of control animals. D: plasma CTGF levels in ob/ob and ob/+ mice. E: urinary CTGF excretion in ob/ob and ob/+ mice. Values in A, B, D, and E are means ± SE.

3B). In consecutive urine samples from the group of 9-wk STZ-DM mice, CTGF levels were significantly increased at all time points (Fig. 3C). Plasma CTGF levels increased >10-fold in three out of five ob/ob mice (Fig. 3D). Urinary CTGF excretion in ob/ob mice increased 15–400 times compared with their non-diabetic ob/+ littermates (Fig. 3E). Plasma CTGF levels and urinary CTGF excretion of control C57BL/6J mice and non-diabetic ob/+ mice were similar.

CTGF in situ hybridization as well as CTGF immunohistochemistry was performed in paraffin sections of renal tissue to determine the localization of CTGF expression. In healthy control animals, some glomerular cells as well as some cells in vessel walls expressed CTGF mRNA, but hardly any renal CTGF protein expression was observed. However, in STZ-DM mice, both renal CTGF mRNA and protein expression were strongly increased (Fig. 4, A and B). In 1-, 2-, and 4-wk STZ-DM mice, CTGF protein appeared mainly localized in podocytes (Fig. 4B). In 9-wk STZ-DM mice, however, in addition to CTGF expression in podocytes, Bowman's capsule and mesangial cells also appeared to be CTGF positive. In these mice, thickening of Bowman's capsule and occasional glomerulosclerosis were observed (Fig. 4B).

Similarly, in ob/+ mice, as in C57BL/6J control mice, hardly any CTGF protein staining was observed (Fig. 4B), whereas in ob/ob mice, CTGF protein expression was increased and mainly localized in podocytes.

To investigate the level of renal CTGF mRNA expression, quantitative PCR was performed on cDNA of (total) renal cortex of STZ-DM and control mice. As early as 2 wk of STZ-DM, CTGF mRNA expression was upregulated threefold and remained at that level until 9 wk (Fig. 5A). Upregulation of TGF-β1 expression, an important inducer of CTGF expression, has been described under diabetic conditions. Therefore, we also determined TGF-β1 mRNA expression in these animals. Renal cortical TGF-β1 expression was increased twofold at 1, 2, and 4 wk after development of diabetes (Fig. 5B). In 9-wk STZ-DM mice, renal TGF-β1 expression increased fourfold.

Quantitative PCR was also performed on other organs of 9-wk diabetic animals. This revealed that CTGF mRNA expression was also significantly increased in the heart and liver but not in the spleen of 9-wk diabetic mice (Fig. 5C). To obtain an impression of the contribution of the kidney to total (systemic) CTGF expression, we multiplied relative CTGF mRNA abundance by organ mass (Fig. 5E). These calculations indicate that, at least in terms of mRNA, the kidneys might contribute up to twice as much as the liver and three times as much as the heart to total CTGF expression. At 9 wk, also the relative and absolute TGF-β1 mRNA expression in the renal cortex, but not in the heart, liver, and spleen, was increased compared with controls (Fig. 5, D and F).

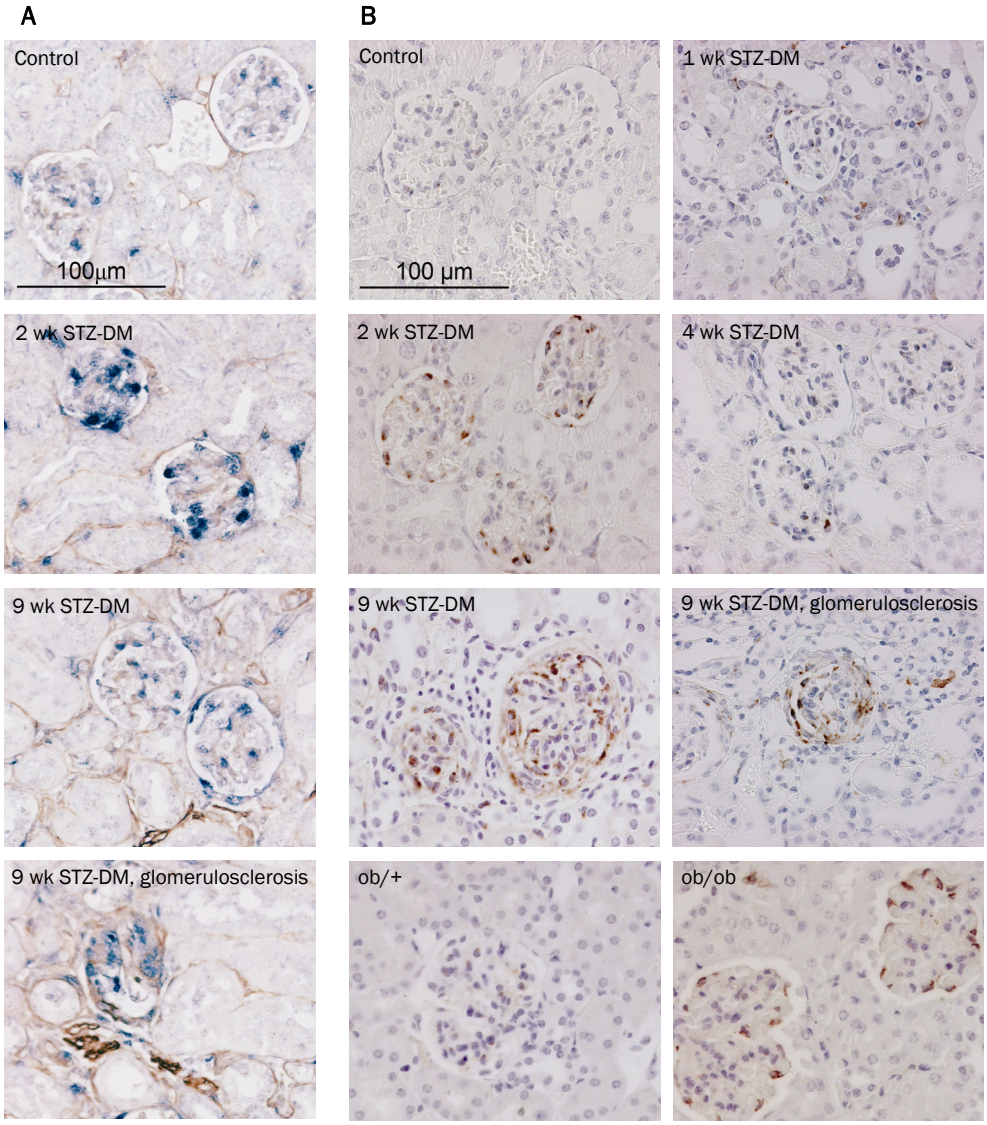

Figure 4: Glomerular CTGF expression in non-diabetic and diabetic mice. A: CTGF mRNA expression was visualized by in situ hybridization (blue staining); as counterstain, collagen IV protein expression was detected by immunohistochemistry (brown). CTGF mRNA is mainly present in glomeruli of STZ-DM mice, as well as in vessel walls and in the tubulo-interstitium. B: CTGF protein expression in non-diabetic and 1-, 2-, 4-, and 9-wk STZ-DM and ob/ob mice detected by immunohistochemistry (brown); sections were counterstained by hematoxylin (blue). CTGF protein is mainly present in glomeruli of STZ-DM and ob/ob mice. Pictures are taken at similar magnification.

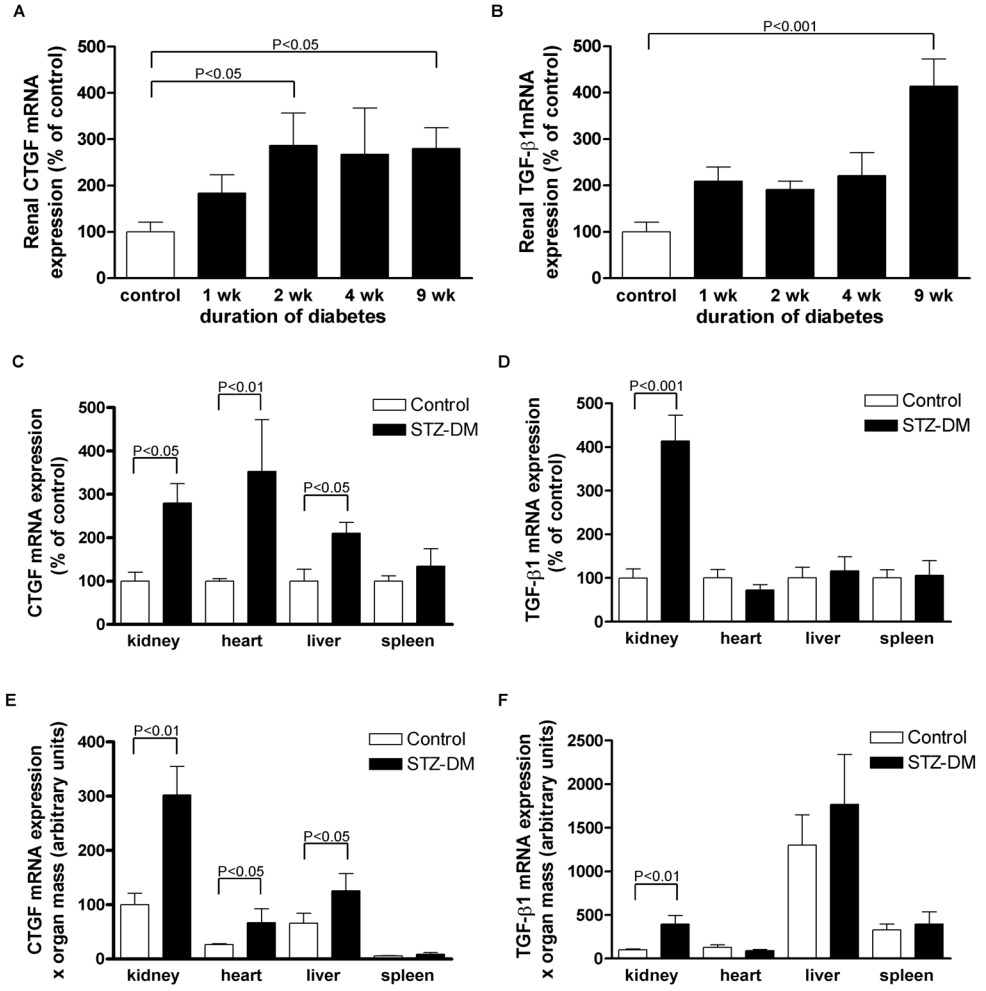

Figure 5: CTGF and transforming growth factor (TGF)-β1 mRNA expression in STZ-DM and control mice. A: total renal cortical CTGF mRNA expression. B: total renal cortical TGF-β1 expression in control and 1-, 2-, 4-, and 9-wk DM mice. C: CTGF mRNA expression in kidney, heart, liver, and spleen of 9-wk diabetic and control mice. D: TGF-β1 mRNA expression in kidney, heart, liver, and spleen of 9-wk diabetic and control mice. E: CTGF mRNA expression corrected for organ mass. F: TGF-β1 mRNA expression corrected for organ mass. Values are means ± SE.

Urinary CTGF level is correlated with nephropathy

One of the main questions of this study was whether CTGF is associated with the development of DN. Because increased albuminuria is the main early characteristic of DN, we assessed the possible correlation between urinary CTGF excretion and albuminuria in the collected urine samples of the STZ-DM mice (1, 2, 4, and 9 wk). Urinary CTGF excretion was significantly correlated with urinary albumin excretion in these animals (Fig. 6, R = 0.86, P < 0.0001). The number of samples from ob/ob mice was too small for such an assessment.

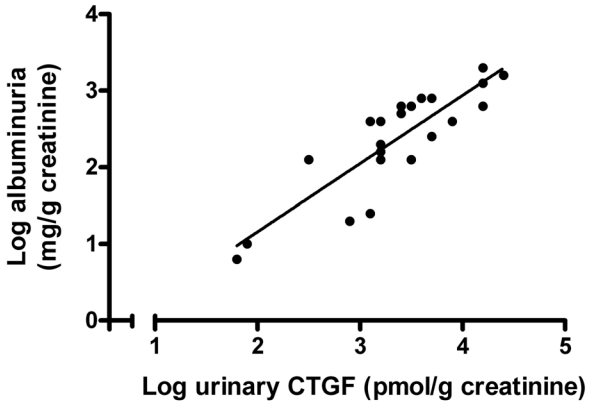

Figure 6: Correlation between urinary CTGF excretion and albuminuria in STZ-DM mice.
$R^2 = 0.74$, $P < 0.0001$: data from 1-, 2-, 4-, and 9-wk diabetic mice are combined.

DISCUSSION

The objective of the present study was to determine the temporal profile of the renal CTGF expression pattern in murine experimental DM and the relationship of plasma, urinary, and renal CTGF levels to the development and severity of DN.

Pancreatic β-cell depletion by STZ injection in C57BL6/J mice has been used extensively as a model for type 1 diabetes and might also be applicable in future studies in genetically modified mice, many of which are on a C57BL6/J background [31]. A disadvantage of this model has been that within a few weeks after induction of DM, most of the animals die from severe wasting and dehydration due to extreme hyperglycemia and insulin deficiency.

We used insulin implants to prevent severe dehydration and maintain sufficient metabolic control for at least 9 wk, despite the persistence of high blood glucose levels and elevated plasma fructosamine levels. With this approach, the mice developed progressive albuminuria, matrix expansion, and elevated plasma creatinine. These disturbances were already present after 1 or 2 wk, demonstrating that, in this model of insulin-treated STZ-DM, the onset of nephropathy is rapid and progressive. STZ has intrinsic toxic effects on the kidney, especially the tubular epithelium. This might be an important confounder when DN in STZ-treated mice is studied. However, it has been established that in rats in which treatment with STZ was followed by pancreatic islet or sham transplantation, increased kidney mass and albuminuria as well as decreased GFR were mainly caused by the diabetic status and not by STZ [32]. Nevertheless, it remains important to control for a possible confounding effect in a study of nephropathy in STZ-induced diabetes. Therefore, we reproduced analyses of key parameters in a genetic model of type 2 DM, hIAPP-transgenic ob/ob mice [33]. This revealed that, although severe hyalinosis and nodular lesions were not observed, the upregulation of CTGF is a phenomenon that occurs in close association with renal deterioration in two independent models of murine diabetes.

CTGF is a key growth factor linked with enhanced ECM expression in numerous physiological and pathological processes. Quantitative RT-PCR and in situ hybridization of renal tissue showed that CTGF mRNA expression was upregulated in the kidneys of STZ-DM mice. This is in agreement with previous studies in other models of DN [15, 17]. However, the spatiotemporal distribution of diabetes-induced CTGF mRNA and protein expression in the kidney has not been resolved in detail [14, 34, 35]. In situ hybridization and immunohistochemistry showed that CTGF expression was increased in kidneys of diabetic mice and mainly localized in the epithelial cells of the glomerulus. After 9 wk of STZ-DM, CTGF was also present in Bowman's capsule and in the mesangial area, in addition to persistent expression in podocytes. Podocytes contribute to synthesis and maintenance of the GBM. Therefore, increased CTGF expression in podocytes might play an important role in diabetes-induced GBM thickening. In vitro,

CTGF importantly contributes to ECM accumulation by rat and human mesangial cells [15, 36]. Cross talk between podocytes and endothelial cells has been described for angiopoietin 1 and VEGF [37]. Similarly, cross talk between podocytes and mesangial cells might also be possible, but it is not clear whether an increase in mesangial matrix in this model of DM relates to CTGF expression by podocytes or to CTGF derived from mesangial and other cells or from the circulation.

Plasma CTGF levels were variably increased in most of the diabetic mice. A comparable increase in plasma CTGF level has been observed in human DN, where this was shown to correlate with albuminuria [25].

We also detected increased urinary CTGF levels in the STZ-DM mice as well as in ob/ob mice, confirming previous observations in experimental DM, as well as in human DN [23, 24]. In the present study, we confirmed that urinary CTGF is correlated with albuminuria, the hallmark of DN, which is consistent with the notion that CTGF plays a pathogenic role in DN.

The markedly increased urinary CTGF excretion in 9-wk STZ-DM mice, and even more in ob/ob mice, together with the strongly increased expression of CTGF in glomerular podocytes suggest that at least part of the urinary CTGF excretion is derived from local renal production. Nevertheless, it remains difficult to identify the main source of the increased urinary CTGF levels.

Our finding that, in STZ-DM, CTGF mRNA expression is upregulated not only in the kidney but also in several other organs (Fig. 4C) and that the plasma CTGF level is upregulated twofold suggests that at least part of the increased urinary CTGF excretion is derived from renal filtration of the relatively small CTGF molecule and possibly fragments thereof. In addition, proteinuria might hamper tubular reabsorption of filtered CTGF from the primary urine, which would independently contribute to higher levels in urine, and provide a trivial explanation for the observed correlation of urinary CTGF with urinary albumin. Arguing against the latter, the fractional excretion of CTGF at 9 wk STZ-DM was about six times higher than the fractional excretion of albumin (data not shown). Still, although less likely in this markedly proteinuric condition, we cannot exclude the possibility that this difference relates, at least in part, to difference in molecular size and maybe charge [molecular mass of albumin is ~66 kDa compared with full-length CTGF (38 kDa) and CTGF NH_2-terminal fragment (18 kDa)]. Hence, the DM-associated increase in urinary excreted CTGF could well be of mixed renal and extrarenal origin. Ultimately, experiments involving injection of labeled CTGF and CTGF fragments will be necessary to better define the contribution of plasma levels and local renal production to urinary CTGF excretion.

Comparing CTGF mRNA levels in normal human tissues by Northern blot analysis, Oemar et al. [38] showed that CTGF expression is more abundant in kidney than in the heart, liver, lung, and pancreas. In control mice, we confirmed by quantitative RT-PCR

that CTGF expression was highest in the kidney, followed by the left ventricular tissue of the heart, than the liver and was lowest in the spleen. In situ hybridization and immunohistochemistry of these tissues showed that in addition to the renal cortex, also the atria, but not the ventricles of the heart abundantly express CTGF mRNA and protein as was reported by Chuva de Sousa Lopes et al. [39]. Overexpression of CTGF in the diabetic heart, vitreous humor, and retina has been reported previously [40-42]. To see whether in our DM models upregulation of CTGF expression is kidney specific or a generalized, systemic phenomenon, we next compared CTGF mRNA levels in the kidney, heart, liver, and spleen of 9-wk STZ-DM and control mice. Besides in the kidney (2.8-fold), an increase in the relative amount of CTGF mRNA was evident in the ventricular tissue of the heart (3.5-fold) and in the liver (2.1-fold) of 9-wk STZ-DM mice. Moreover, correction for organ mass indicates that the kidneys might contribute approximately twice as much as the liver and three times as much as the heart to total (systemic) CTGF mRNA expression under diabetic conditions. Together, these data indicate that CTGF overexpression plays a central role in DM, with involvement not only in the development of DN but also in the systemic complications of DM.

TGF-β, one of the main inducers of CTGF, is considered an important profibrotic growth factor in the development and progression of DN [1]. It is known to be overexpressed in mesangial and endothelial cells of diabetic glomeruli and has been implicated in GBM thickening and albuminuria, both characteristics of DN [1]. However, more recently, factors relevant to the DM-associated pathogenic processes of hyperglycemia and hypertension have been reported to stimulate expression of CTGF and ECM [17, 43, 44]. This can occur in a TGF-β-independent manner. For example, in mouse podocytes, it has been shown that the high glucose-induced expression of the α1- and α5-subunits of collagen type IV is independent of TGF-β1 [22]. In renal fibroblasts, TGF-β1-independent induction of collagen IV and fibronectin expression under high-glucose conditions has been described [45, 46]. Furthermore, in rats with unilateral ureteral obstruction, CTGF antisense oligonucleotide treatment reduced the gene expression of CTGF, fibronectin, and fibronectin ED-A, whereas TGF-β1 gene upregulation was not affected. In this study, the deposition of these proteins was also reduced by the CTGF antisense treatment [47].

In our study, TGF-β1 mRNA expression was increased fourfold in renal cortex of 9-wk STZ-DM mice, but in diabetic heart, liver, and spleen the TGF-β1 level was not different from controls. This suggests that, at least in the latter organs, CTGF overexpression occurs independently of increased TGF-β1 mRNA expression levels. However, it does not preclude a critical role of TGF-β1, because the abundance and signaling activity thereof are largely regulated at the level of translation and conversion from a latent to the active form [48]. Alternatively, other factors and processes that are responsible for

TGF-β-independent stimulation of CTGF gene expression could mediate the observed increase in heart and liver CTGF.

Glomerular overexpression of other growth factors in DN might act in concert with increased CTGF to promote pathogenesis. For example, it is known that VEGF is highly expressed in diabetic podocytes and that inhibition of VEGF in these cells attenuates glomerular hypertrophy, GBM thickening, and mesangial matrix expansion [49, 50]. PDGF-B and its receptor are highly expressed in mesangial cells and podocytes of diabetic rats [19], and IGF-I is yet another growth factor implicated in matrix accumulation in DN. Both CTGF and IGF-I are produced by human renal fibroblasts and cooperate in the induction of collagen production by high glucose [8]. It is known that CTGF, as a matricellular protein, can physically interact with several of these and other growth factors [9]. In this way, CTGF can promote TGF-β1 signaling and inhibit the action of BMP4 [51]. CTGF can also inhibit VEGF-induced angiogenesis [52], and cooperation of CTGF and IGF-I in the induction of collagen I and -III secretion has been demonstrated in human kidney fibroblasts [46]. Thus CTGF can function as a cofactor for other growth factors and modulate their biological activity in a way that contributes to the development of DN. Moreover, recent evidence indicates that, in addition to its role as a modulator of ECM, CTGF can signal through direct binding to the neurotrophin receptor TrkA [53] and through interaction with low-density lipoprotein receptor-related protein [54-56].

Different fragments of the CTGF protein have been detected in vitro and in vivo, and at least some of these have biological activity. The CTGF molecule, which consists of four modules, is mostly cleaved between modules II and III, yielding fragments of 16–20 kDa, but smaller fragments have also been identified [57, 58]. Grotendorst et al. [59] reported distinct biological effects of CTGF fragments; the NH_2-terminal part of CTGF mediates myoblast formation and collagen synthesis, whereas the COOH-terminal part is involved in fibroblast differentiation. However, the forms in which CTGF is present in plasma, urine, and tissue are still mainly undefined, and in addition our assays did not allow such specification.

In summary, we have shown that CTGF levels are increased in plasma, urine, and renal tissue of diabetic mice in already a very early phase of nephropathy. Moreover, the observed association of levels and distribution pattern of CTGF with markers of nephropathy lends further support to its proposed role as an early marker and key pathogenic factor in DN.

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Chapter 6

Connective tissue growth factor determines albuminuria and GBM thickening in experimental diabetic nephropathy.
Involvement of reduced MMP activity?

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Submitted

ABSTRACT

We investigated whether altered expression levels of the pro-fibrotic cytokine connective tissue growth factor (CTGF) might affect albuminuria, GBM thickening, GBM matrix synthesis, and the activity of matrix metalloproteinases (MMPs) in streptozotocin induced diabetes in mice lacking one functional CTGF allele ($CTGF^{+/-}$ mice). $CTGF^{+/+}$ littermates served as controls.

Albuminuria in diabetic $CTGF^{+/-}$ mice was less severe than in diabetic $CTGF^{+/+}$ mice. In addition, considerable GBM thickening (+17%) present in diabetic $CTGF^{+/+}$ mice, was absent in diabetic $CTGF^{+/-}$ mice. Renal cortical mRNA expression was elevated 3-fold in diabetic $CTGF^{+/+}$ mice as compared to non-diabetic $CTGF^{+/+}$ mice. However, in diabetic $CTGF^{+/-}$ mice, mean increase was only 1.5-fold (not significant). Collagen IVα1, fibronectin and fibronectin ED-A were upregulated in diabetic mice as compared to controls, but not significantly different in diabetic $CTGF^{+/-}$ vs. diabetic $CTGF^{+/+}$ mice. In contrast, MMP activity was decreased by approximately 40% in diabetic $CTGF^{+/+}$ mice, but not in diabetic $CTGF^{+/-}$ mice.

Thus, in this mouse model of type 1 DM, GBM thickening and albuminuria were significantly attenuated by an experimental intervention that limited diabetes-induced increases in renal cortical CTGF expression, in association with reduced MMP activity.

INTRODUCTION

In diabetic nephropathy (DN), glomerular basement membrane (GBM)-thickness correlates with severity of proteinuria [1]. The balance between production and degradation of GBM constituents determines the actual GBM thickness. Both production and degradation are, at least in part, effectuated by podocytes. In DN, the composition of the GBM is altered, and GBM synthesis is increased, while activity of matrix degrading metalloproteinases (MMPs) is decreased, possibly due to increased levels of tissue inhibitors of metalloproteinases (TIMPs) [2].

In the adult glomerulus it has been shown that the podocytes add and assemble GBM matrix molecules [3]. Both GBM matrix constituents and, matrix degrading proteases and their inhibitors, including MMP-2 and TIMPs, are expressed by podocytes [4]. The major determinants of altered regulation of GBM-synthesis and –degradation in DN remain largely unknown, although it seems likely that growth factors are critically involved.

Previously, we and others [5-12] have shown that expression level of connective tissue growth factor (CTGF, CCN-2) in the kidney, urine, and plasma is increased in human as well as experimental diabetes, in particular in association with DN, and that (excess) renal CTGF is mainly localized in podocytes [10]. This specific regulation and localization, together with its role in matrix regulation, have identified CTGF as a key-factor in development and progression of DN. CTGF, a 36–38 kD secreted protein, is strongly upregulated in many fibrotic disorders. [9, 10, 12]. Its expression is induced by TGF-β stimulation, but TGF-β-independent upregulation of CTGF has also been reported [13, 14]. CTGF expression is also induced by high glucose levels and advanced glycation end products (AGEs) [9, 15-17].

However, whether CTGF is critical for the characteristic changes in the expression of ECM proteins and of proteins involved in matrix degradation (MMPs and TIMPs) under diabetic conditions is unknown [18, 19].

In the present study, we have further investigated the involvement of CTGF in diabetic nephropathy. For this, we induced diabetes in heterozygous $CTGF^{+/-}$ mice and their wild type ($CTGF^{+/+}$) littermates (homozygous $CTGF^{-/-}$ mice are not viable [20]). $CTGF^{+/-}$ mice had no obvious spontaneous phenotype. However, after induction of diabetes, heterozygotes expressed significantly less CTGF, which identified them as a suitable tool to study the impact of an experimentally imposed limitation on CTGF availability on the development and severity of diabetes-induced nephropathy.

The results obtained in this model indicate that both albuminuria and GBM thickening in DM are critically dependent on increased CTGF expression and provide evidence for associated decrease in MMP activity as a mediator of CTGF action.

RESEARCH DESIGN AND METHODS

Animals

CTGF heterozygous ($CTGF^{+/-}$) BALBc/129Sv mice in which exon 1 of one of the CTGF alleles has been exchanged by a neomycin resistance gene resulting in a CTGF null allele, have been described by Ivkovic et al [20]. Male BALBc/129Sv $CTGF^{+/-}$ mice were crossbred with CTGF wild type female C57Bl/6J mice (Harlan, Horst, The Netherlands). The females of the F1 offspring were used for the present study. The mice were genotyped and divided into 4 groups: control $CTGF^{+/+}$, control $CTGF^{+/-}$, diabetic $CTGF^{+/+}$ and diabetic $CTGF^{+/-}$. Diabetes was induced at 16 weeks of age, by means of a single i.p. injection of streptozotocin (STZ, Sigma, St. Louis, MO, USA), 200 mg/kg dissolved in 100 mM sodium citrate buffer (pH 4.6). Control animals were injected with sodium citrate buffer alone. All animals were housed in a room with constant temperature, on a 12 hr light/12 hr dark cycle and allowed a standard pellet laboratory chow and water ad libitum. Induction of diabetes was determined 3 days after injection by measurement of blood glucose levels (Medisense Precision Xtra apparatus, Abbott, Bedford, USA). Slow release insulin pellets (Linshin, Scarborough, Canada) were used in diabetic mice to stabilize the condition of the animals for at least 17 weeks. After 9 weeks of diabetes unilateral nephrectomy was performed on all animals to accelerate the development of nephropathy.

After 17 weeks of diabetes urine samples were collected, the animals were sacrificed, blood was collected in EDTA and plasma was stored at -70°C. Tissues were frozen in liquid nitrogen or fixed in formaldehyde (4%) and embedded in paraffin. The experiments were performed with the approval of the Animal Ethical Commission of the University of Utrecht.

Plasma and urine determinations

HbA_{1c} was determined by means of an immunochemical method (Tina-quant; Roche/Hitache, Mannheim, Germany). Albumin levels in urine were determined by means of sandwich ELISA using a goat-anti-mouse albumin antibody (Bethyl Laboratories, inc., Montgomery, TX, USA). Urinary creatinine and plasma urea concentrations were determined by enzymatic assays (J2L Elitech, Labarthe Inard, France).

CTGF ELISA on plasma and urine samples

CTGF levels in plasma and urine were determined by means of sandwich ELISA using 2 distinct specific antibodies both directed against the CTGF protein (FibroGen, South San Francisco, CA, USA), as was described previously [10]. This assay detects full-length as well as N-terminal fragments of CTGF. To avoid confusion due to differences

in the molecular weight of full-length and different fragments of CTGF, all levels were expressed as pmol/l.

CTGF Immunohistochemistry

4 µm sections were cut from formalin fixed and paraffin embedded tissue samples and mounted on silan-coated slides. Slides were fixed overnight at 56˚C in an incubator. Sections were deparaffinized and rehydrated. A 3-minute incubation of the slides at 37˚C with Protease XXIV (Sigma) 4.4 units/ml phosphate) was performed to retrieve the epitopes. After 2 minutes of washing with distilled water, slides were treated for 20 minutes with a 1,5% hydrogen peroxide solution in PBS to block endogenous peroxidase activity. Tissue sections were washed three times 3 minutes in PBS-Tween and incubated for one hour with a CTGF specific human monoclonal antibody (FG-3019, FibroGen, 8 µg/ml), in PBS/1%BSA. Sections were rinsed three times 3 minutes in PBS-Tween and incubated for 30 minutes with rabbit-anti-human IgG (DakoCytomation A/S, Copenhagen, Denmark), diluted 1:1500 in PBS/1%BSA containing 5% normal mouse serum (DakoCytomation). After three times 3 minutes of washing with PBS-Tween, sections were incubated with goat-anti-rabbit Powervision-PO for 30 minutes followed by three times 3 minutes of washing with PBS-Tween. Subsequently the sections were developed with Nova RED (Vector Laboratories, Burlingame, CA, USA) and rinsed with distilled water. Nuclear staining was performed with haematoxylin and developed in running tab water for 10 minutes. Tissue sections were dehydrated and covered with pertex and a coverslip.

Electron microscopy (EM)

Tissue samples were fixed in Karnovsky solution and stored at 4˚C. Upon embedding, tissue samples were rinsed 2 times with 0.1 M Na-cacodylatebuffer, pH 7.4 followed by a two hour fixation with 1% osmiumtetroxide, dehydrated with acetone and embedded in epon. Ultrathin sections of 95 nm were cut and mounted on copper one-hole specimen support grids and dried to the air. Sections were stained with uranyl acetate and lead citrate to provide the contrast necessary to reveal details of the cells ultrastructure.

All ultra thin sections were photographed using a transmission electron microscope (JEM-1200 EX, JEOL, Peabody, MA, USA). Thickness of the GBM was based on measurements in sections of 5 random glomeruli per mouse. From each glomerulus, 5 pictures covering the whole glomerulus were taken at a magnification of 5000x and analyzed by computer image analysis. Basement membrane thickness was established with the computer program ImageJ (NIH, http://rsb.info.nih.gov/ij/) at sites where the basement membranes were cut perpendicularly.

ECM protein immunohistochemistry

HSPG content was assessed using mouse monoclonal anti-heparan sulphate (JM403) [21]; Hamster monoclonal MI91 was used to detect anti-agrin core protein [22]; rabbit-anti-mouse collagen IV was from Biodesign international (Maine, USA) and, rabbit-anti-mouse E2 EHS laminin was produced at the Nephrology Research Laboratory, Nijmegen Centre for Molecular Life Sciences, The Netherlands. Sections were embedded in Vectashield mounting medium H-1000 (Vector Laboratories Inc., Burlingame, CA) and examined on a Zeiss Axioscope (equipped with an epi-illuminator and Nikon coolpix DXM 1200 camera).

Quantitative RT-PCR

The mRNA levels of CTGF, TGF-β1 and several ECM components in total renal cortex were quantified by real-time quantitative PCR. For this purpose RNA was isolated from frozen sections of renal cortex using the RNeasy minikit (Qiagen, Hilden, Germany). cDNA synthesis was performed on 3 µg of RNA using oligo-$dT_{12\text{-}18}$ and Superscript reverse transcriptase (Invitrogen, Carlsbad, CA, USA).
Quantitative RT-PCR was performed using gene specific Assay on Demand (Applied Biosystems, Foster City, Canada) according to the manufacturers instructions and was analysed on an ABI PRISM 7900 Sequence Detector System (Applied Biosystems). The thermal cycling comprised a denaturation step at 95 °C for 10 minutes followed by 45 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Quantitative values were obtained from the threshold PCR cycle number, at which the increase in signal associated with an exponential growth for PCR product, can be detected. The relative mRNA levels in each sample were normalized for the TATA box-binding Protein (TBP) and β-actin contents.

In situ zymography

Glomerular MMP activity was visualized by in situ zymography and confocal laser-scanning microscopy. Frozen tissue sections (6 µm) were incubated with DQ™ gelatin from pig skin (Molecular probes), 1:20 diluted in 50 mM Tris-HCl buffer containing 10 mol/l $CaCl_2$, 0.05% Brij 35 and 5 mmol/l PMSF, pH 7.4 (100 µl/slide). Slides were incubated in a dark humidified chamber at 37 ºC for 19 hrs. 1,10-phenanthroline monohydrate (2 µg/ml), a MMP inhibitor was used to verify that the obtained gelatinase activity specifically represented MMP activity. Nuclei were counterstained in red with propidium iodide.

Gelatinase activity assay

From frozen kidneys, 10 sections of 20 µm were cut and dissolved in lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, pH 7.4). Gelatinase activity was measured

with an EnzChek Gelatinase assay kit using the protocol of the manufacturer (Molecular Probes). Collagenase was used as standard and specificity of the gelatinase activity was verified using the MMP inhibitor 1,10-phenanthroline monohydrate. Protein concentrations of the lysates were determined colorimetrically (Pierce) and used for normalization of collagenase activity per lysate.

Statistical analysis

Data are expressed as means ± SD or median (range), when appropriate. Statistical analysis was performed using two-way ANOVA followed by a Bonferroni post-hoc test. Non-parametric data were ranked prior to analysis. Differences between groups were considered statistically significant when $P < 0.05$.

Table 1: General characteristics of diabetic and control animals.

Parameters	Control		Diabetic		ANOVA (2-way)	
	CTGF $^{+/+}$	CTGF $^{+/-}$	CTGF $^{+/+}$	CTGF $^{+/-}$	DM	Genotype
N	4	5	4	5		
BW day 0 (g)	22.7 ± 2.0	22.7 ± 1.5	22.7 ± 0.7	23.4 ± 1.7	NS	NS
BW at sacrifice (g)	27.9 ± 3.4	28.1 ± 1.3	24.2 ± 1.9	24.7 ± 1.3†	<0.01	NS
BW sacr/BW day 0 (%)	123 ± 9	124 ± 6	107 ± 5*	106 ± 10†	<0.001	NS
Right-KW (mg)	203 ± 11	209 ± 9	425 ± 102*	356 ± 78†	<0.001	NS
R-KW/BW (%)	0.73 ± 0.05	0.74 ± 0.01	1.75 ± 0.39*	1.45 ± 0.32†	<0.001	NS
Blood glucose (mmol/l)	7.4 ± 1.5	7.5 ± 0.9	> 25	> 25		
HbA_{1c} (%)	4.7 ± 0.2	4.8 ± 0.2	9.2 ± 1.6*	8.1 ± 1.2†	<0.001	NS
Albuminuria (mg/g creatinine)	46 ± 22	35 ± 18	176 ± 26*	111 ± 39†,‡	<0.05	<0.001
Plasma urea (mmol/l)	8.6 ± 0.8	8.1 ± 0.8	14.1 ± 3.7*	14.9 ± 2.0†	<0.001	NS

Means ± SD, * $P < 0.05$ vs. control CTGF $^{+/+}$, † $P < 0.05$ vs. control CTGF$^{+/-}$, ‡ $P < 0.05$ vs. diabetic CTGF $^{+/+}$.

RESULTS

Induction of diabetes

Three days after injection, all STZ-treated mice were diabetic, as evidenced by elevated blood glucose concentrations. At the end of the experiment, 17 wk after STZ, HbA_{1c}, kidney mass, albuminuria and plasma urea levels were significantly increased compared to controls, indicating development of DN (table 1). There were no significant differences between control $CTGF^{+/+}$ and control $CTGF^{+/-}$ mice. All parameters for diabetes and DN were significantly increased in STZ-treated $CTGF^{+/+}$ as well as $CTGF^{+/-}$ mice. However, albuminuria was significantly less pronounced in diabetic $CTGF^{+/-}$ mice than in diabetic $CTGF^{+/+}$ mice. Also increase of kidney weight ("hypertrophy") and HbA_{1c} tended to be less severe in diabetic $CTGF^{+/-}$ mice compared to diabetic $CTGF^{+/+}$ mice, but these differences did not reach statistical significance (P=0.19, and P=0.41, respectively).

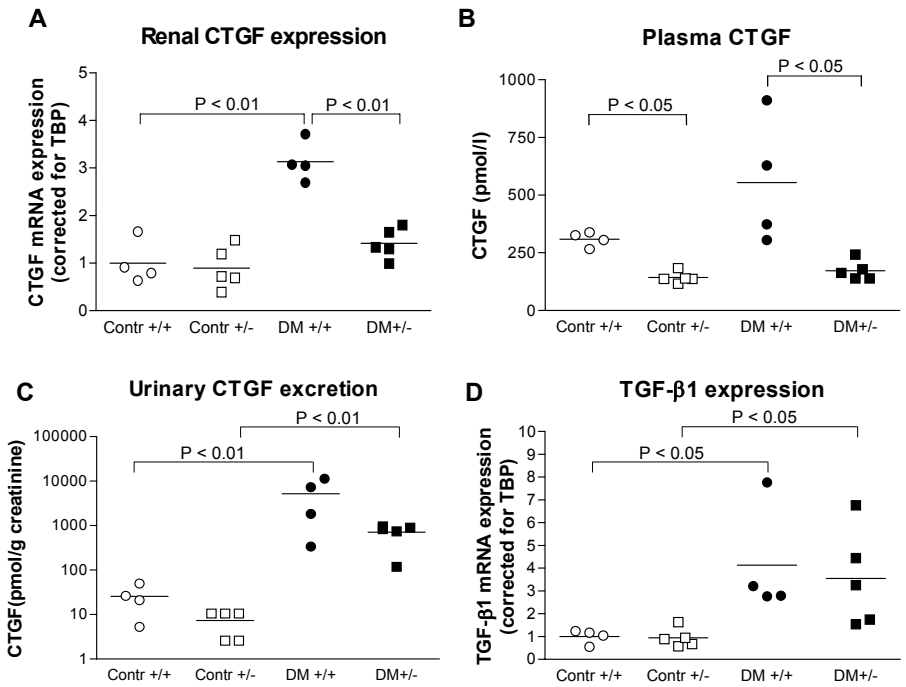

Figure 1: CTGF levels in plasma, urine and renal tissue. A: CTGF mRNA expression levels in total renal cortex of diabetic and control animals. CTGF expression was 3-fold increased in renal cortex of diabetic $CTGF^{+/+}$ mice compared to control $CTGF^{+/+}$ mice. In diabetic $CTGF^{+/-}$ mice CTGF expression level was almost at control level (2-way ANOVA: DM P<0.001, genotype P<0.001). B: Plasma CTGF levels were significantly reduced in $CTGF^{+/-}$ mice as compared to $CTGF^{+/+}$ mice (two-way ANOVA: DM P< 0.05, Genotype P < 0.001). C: Urinary CTGF excretion was increased in diabetic animals as compared to non-diabetic controls (2-way ANOVA: DM P< 0.05, genotype NS). D: TGF-β1 mRNA expression in total renal cortex was increased in diabetic mice compared to controls (2-way ANOVA: DM P<0.01, genotype NS).

CTGF expression

Renal CTGF mRNA expression was determined by means of Q-PCR (fig 1a). This showed that CTGF mRNA expression was 3-fold increased in total renal cortex of DM CTGF$^{+/+}$ mice as compared to control CTGF$^{+/+}$ mice. However, in DM CTGF$^{+/-}$ mice, mean CTGF mRNA expression was increased only 1.5-fold (i.e. 50% of that in DM CTGF$^{+/+}$), which was not significantly different from controls (P=0.095). Plasma CTGF was higher in CTGF$^{+/+}$ mice than in CTGF$^{+/-}$ mice and this difference was accentuated after 17 wk of diabetes (fig 1b). Urinary CTGF excretion was markedly
increased in both diabetic CTGF$^{+/+}$ and diabetic CTGF$^{+/-}$ mice. Although urinary CTGF in diabetic CTGF$^{+/-}$ mice appeared to be lower than in diabetic CTGF$^{+/+}$ mice, this difference did not reach statistical significance (P=0.29, fig 1c; note log scale).
TGF-β1 mRNA expression, was similarly increased in both diabetic CTGF$^{+/+}$ and diabetic CTGF$^{+/-}$ mice (fig 1d).
CTGF IHC showed more prominent glomerular CTGF staining in diabetic CTGF$^{+/+}$ mice than in control mice and in diabetic CTGF$^{+/-}$ mice (fig 2). This is in agreement with plasma CTGF levels and renal mRNA expression data for CTGF, which also indicate that lack of one functional CTGF allele resulted in significant impairment of the elevation of CTGF levels in diabetic CTGF$^{+/-}$ animals.

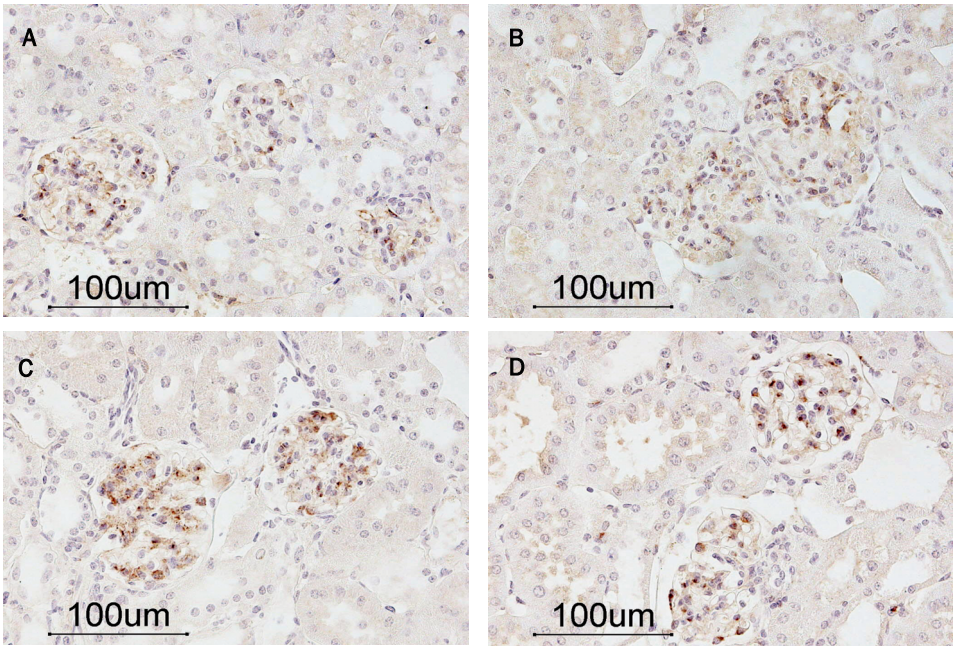

Figure 2: Renal CTGF protein expression. Photomicrographs of IHC staining for CTGF. A: Control CTGF$^{+/+}$, B: Control CTGF$^{+/-}$, C: Diabetic CTGF$^{+/+}$, and D: Diabetic CTGF$^{+/-}$. CTGF staining is more prominent in diabetic CTGF$^{+/+}$ animals compared to control mice and diabetic CTGF$^{+/-}$.

GBM thickness

Examination of ultra-thin tissue sections by transmission electron microscopy showed that the thickness of the GBM in diabetic CTGF$^{+/+}$ mice was increased compared to control CTGF$^{+/+}$ mice (mean increase +17%, p<0.01, fig 3). However, no increase in GBM thickness was observed in diabetic CTGF$^{+/-}$ mice.

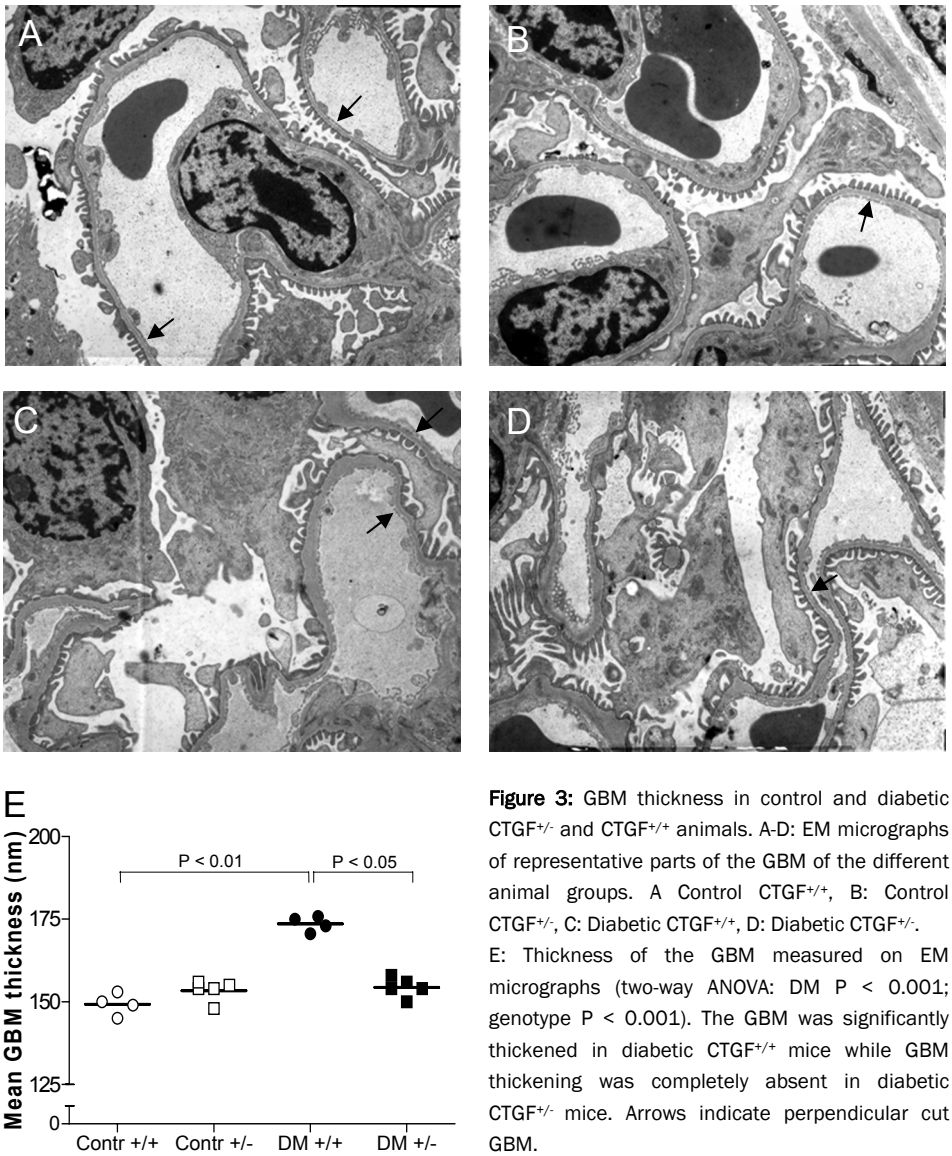

Figure 3: GBM thickness in control and diabetic CTGF$^{+/-}$ and CTGF$^{+/+}$ animals. A-D: EM micrographs of representative parts of the GBM of the different animal groups. A Control CTGF$^{+/+}$, B: Control CTGF$^{+/-}$, C: Diabetic CTGF$^{+/+}$, D: Diabetic CTGF$^{+/-}$.
E: Thickness of the GBM measured on EM micrographs (two-way ANOVA: DM P < 0.001; genotype P < 0.001). The GBM was significantly thickened in diabetic CTGF$^{+/+}$ mice while GBM thickening was completely absent in diabetic CTGF$^{+/-}$ mice. Arrows indicate perpendicular cut GBM.

Expression of GBM matrix proteins and mRNAs

The mRNA expression of selected GBM matrix genes as measured by quantitative RT-PCR (table 2) was not different in control $CTGF^{+/+}$ as compared to $CTGF^{+/-}$ mice. In diabetic mice, renal expression of fibronectin, fibronectin-EDA, collagen IVα1 tended to be higher than in control mice, while the mature collagen-IVα3 and collagen-IVα5 chains were similarly expressed in control and diabetic mice. Again there was no significant difference in diabetic $CTGF^{+/-}$ as compared to diabetic $CTGF^{+/+}$ mice (table 2).

Also at the protein level (as determined by immunofluorescence scores), no significant difference of collagen IV, and laminin in the GBM was observed between diabetic $CTGF^{+/-}$ as compared to diabetic $CTGF^{+/+}$ mice (table 3). Scores for heparan sulphate proteoglycan and agrin appeared unaltered as well (not shown).

Table 2: Renal cortical mRNA expression of ECM constituents, MMPs and TIMP-1 as determined by quantitative RT-PCR. Data (median (range)) are expressed relative to mean of control $CTGF^{+/+}$ mice.

	Control		Diabetic		ANOVA (2-way)	
	$CTGF^{+/+}$	$CTGF^{+/-}$	$CTGF^{+/+}$	$CTGF^{+/-}$	DM	Genotype
GBM matrix genes						
Fibronectin	0.86 (0.28-2.01)	0.63 (0.28-5.35)	2.64 (1.75-6.06)	4.15 (2.46-6.17)	NS	NS
Fibronectin ED-A	0.84 (0.53-1.79)	1.19 (0.71-1.95)	5.1 (1.50-15.10)	6.53 (2.14-22.65) †	<0.05	NS
Collagen IVα1	1.00 (0.83-1.15)	0.78 (0.56-1.21)	2.46 (1.10-2.47)	2.32 (1.50-3.19) †	<0.001	NS
Collagen IVα3	0.83 (0.68-1.67)	0.95 (0.49-1.39)	0.84 (0.22-1.23)	0.48 (0.19-0.78)	NS	NS
Collagen IVα5	0.90 (0.81-1.37)	0.72 (0.47-2.05)	0.91 (0.51-1.98)	0.87 (0.55-2.73)	NS	NS
Genes involved in GBM degradation						
MMP-2	1.07 (0.74-1.11)	1.04 (0.94-1.09)	7.24 (3.82-15.22) *	12.20 (7.52-21.79) †	<0.001	NS
MMP-9	0.92 (0.59-1.57)	1.04 (0.66-1.33)	3.72 (0.74-14.07)	3.43 (1.90-5.33) †	<0.05	NS
TIMP-1	0.91 (0.64-1.54)	0.90 (0.47-1.22)	33.25 (14.10-86.25) *	24.28 (17.48-63.02) †	<0.001	NS

* $P < 0.05$ compared to control $CTGF^{+/+}$, † $P < 0.05$ compared to control $CTGF^{+/-}$.

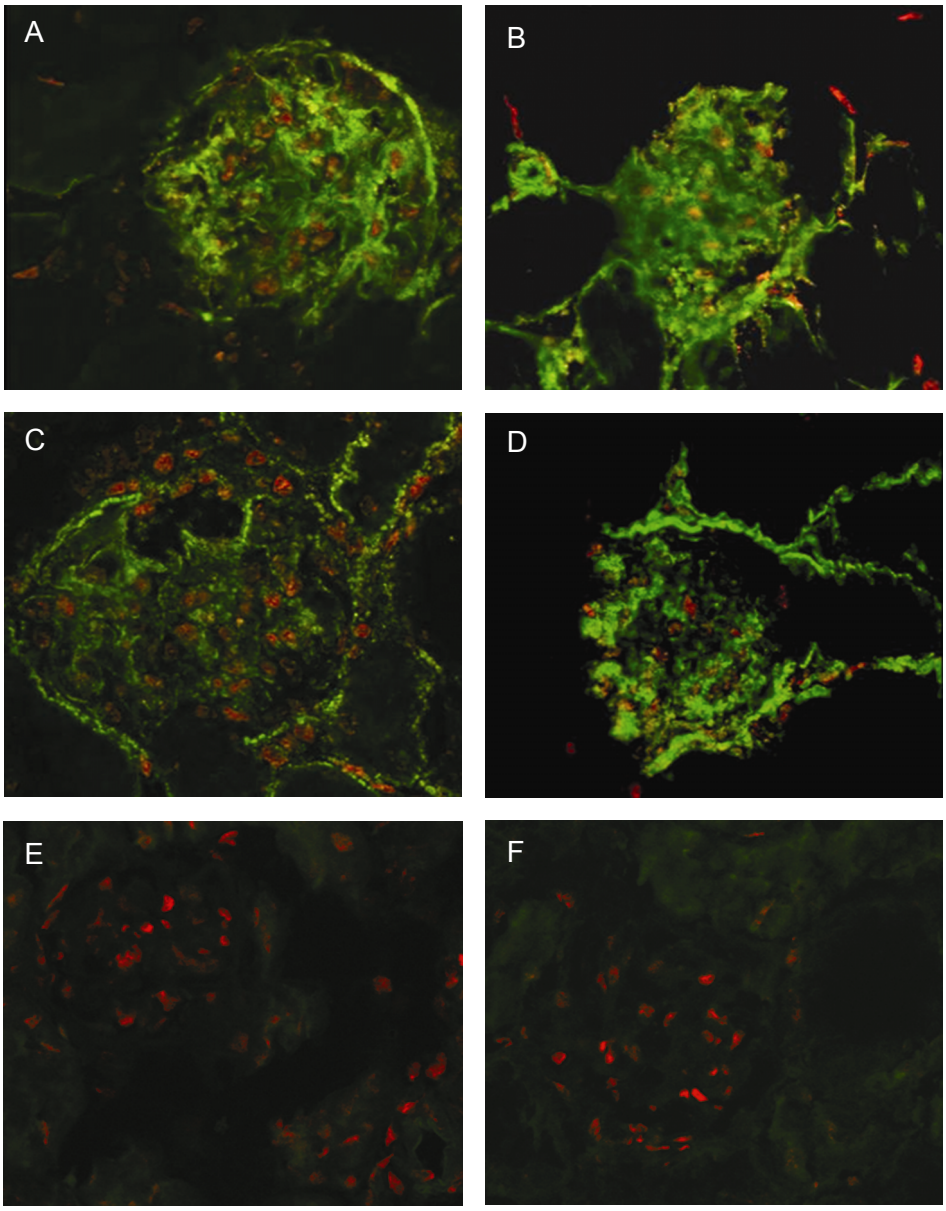

Figure 4: Visualization of gelatinase activity by means of in situ zymography on frozen kidney sections. Gelatinase activity was present in glomeruli of all animal groups and could completely be inhibited with 1,10-phenanthroline monohydrate. A: Control $CTGF^{+/+}$, B: Control $CTGF^{+/-}$, C: Diabetic $CTGF^{+/+}$, D: Diabetic $CTGF^{+/-}$, E: Control $CTGF^{+/+}$ with 1,10-phenantroline monohydrate, F: Diabetic $CTGF^{+/+}$ with 1,10-phenanthroline monohydrate. Green: gelatinase activity, red: nuclei stained with propidium iodide.

MMP-2, MMP-9 and TIMP1 mRNA expression

The mRNA expression of the matrix degrading protease MMP-2, MMP-9 and their inhibitor TIMP-1 were increased significantly in the two diabetic groups compared to the control groups (except for MMP-9 in diabetic $CTGF^{+/+}$ which showed a very large variation; table 2). MMP-9 and TIMP-1 expression levels were similar in diabetic $CTGF^{+/+}$ and diabetic $CTGF^{+/-}$ mice, while MMP-2 expression tended to be higher in diabetic $CTGF^{+/-}$ than in diabetic $CTGF^{+/+}$ mice (NS, P= 0.19).

Table 3: Glomerular protein expression of GBM proteins was assessed by semiquantitative immunofluorescence scoring. Data (means +/- SD) are expressed relative to mean score in control$^{+/+}$ mice.

	Control		Diabetic		ANOVA (2-way)	
GBM protein	$CTGF^{+/+}$	$CTGF^{+/-}$	$CTGF^{+/+}$	$CTGF^{+/-}$	DM	Genotype
Collagen IV	1.00 ± 0.06	1.42 ± 0.28	1.82 ± 0.36	1.41 ± 0.42	<0.05	NS
Laminin	1.00 ± 0.34	1.32 ± 0.25	1.52 ± 0.37	1.82 ± 0.06 †	<0.05	NS
Heparan sulphate	1.00 ± 0.14	0.82 ± 0.52	0.73 ± 0.37	0.87 ± 0.31	NS	NS
Agrin	1.00 ± 0.17	0.99 ± 0.14	1.05 ± 0.08	1.00 ± 0.26	NS	NS

† $P < 0.05$ compared to control $CTGF^{+/-}$.

Gelatinase activity

Gelatin proteolysis (representing activity of MMP-2 and -9) was visualized by means of in situ zymography (ISZ) on frozen kidney sections and quantitative fluorescent detection on renal lysates.

In all groups, in situ zymography (ISZ) showed that gelatinase activity was present mainly in the glomeruli. The activity detected by ISZ was inhibited completely by the general MMP inhibitor 1,10 phenanthroline monohydrate (fig 4). Fluorescent detection in renal lysates showed that the gelatinase activity was approximately 40% decreased in diabetic $CTGF^{+/+}$ mice ($P<0.05$). In contrast, in diabetic $CTGF^{+/-}$ mice, the gelatinase activity was preserved at the same level as in control mice (fig 5).

DISCUSSION

The pro-fibrotic growth factor CTGF is involved in ECM accumulation in DN and many other fibrotic diseases [23-25]. Targeting of CTGF as a therapy for diabetic nephropathy has been proposed by several investigators [18, 23, 26]. We show here, in a genetic model, that an experimentally induced limitation of CTGF expression to an approximately normal level resulted in significantly reduced albuminuria, and completely prevented thickening of the GBM under diabetic conditions in mice. Our data in renal cortex and podocytes indicate that GBM thickening in diabetic mice is CTGF-dependent and is associated with decreased MMP activity. These results are consistent with prior results in human mesangial cells and diabetic rat renal cortex [19].

CTGF expression in $CTGF^{+/-}$ mice, was not increased or only marginally increased under diabetic conditions, while diabetic $CTGF^{+/+}$ littermates exhibited a significant increase in CTGF mRNA as well as CTGF protein content. Remarkably, the diabetes-induced

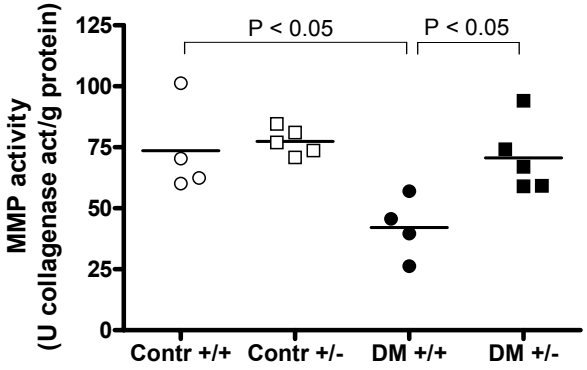

Figure 5: Gelatinase activity measured in renal lysates was significantly reduced in diabetic $CTGF^{+/+}$ mice but was preserved in diabetic $CTGF^{+/-}$ mice (two-way ANOVA: DM $P<0.01$, genotype $P<0.05$).

albuminuria was significantly attenuated, and the GBM thickening observed in $CTGF^{+/+}$ mice was completely prevented in $CTGF^{+/-}$ mice. Consistent with these observations, Usinger et al. recently presented data that administration of a CTGF-neutralizing antibody to db/db mice for 2 months, significantly attenuated diabetes-induced albuminuria and GBM thickening [27]. CTGF-antibody treatment of db/db mice had additional beneficial effects, reducing renal hypertrophy, hyperfiltration and HbA_{1c}, which may have been attenuated also in the present diabetic $CTGF^{+/-}$ mice, although without reaching statistical significance in our relatively small sample size.

Due to the effects of high glucose concentrations and AGEs, the thickness, composition and electrical charge of the GBM changes in diabetes, resulting in leakage of albumin and other proteins into the glomerular filtrate [28]. GBM thickening in DN reflects qualitative changes as well as quantitative imbalance between production and degradation of extracellular matrix components. Increased expression of collagen IV [29], fibronectin [16] and several other matrix components has been observed in podocytes and other glomerular cells under diabetic conditions, and contributes to thickening of the GBM in DN [18]. On the other hand (experimental) diabetes is known to be associated with impaired MMP activity [20] and also several MMPs have been detected in podocytes under control and high glucose conditions [30]. CTGF is involved in regulation of ECM genes as well as MMPs and their inhibitors [19, 31].

High glucose and AGEs induce CTGF expression in renal cells, which might thus be an important intermediate step in development and progression of diabetic nephropathy. In this respect, the contribution of podocytes to increased GBM thickness and permeability is of particular interest because these cells are the major source of renal CTGF overexpression in diabetes [6, 10].

In the present study, diabetic $CTGF^{+/-}$ and diabetic $CTGF^{+/+}$ mice displayed similar increase of the renal cortical expression of mRNAs encoding GBM constituents, despite the fact that significant increase of CTGF expression occurred only in the $CTGF^{+/+}$ diabetic mice and not in the $CTGF^{+/-}$ diabetic mice. Since many reports demonstrate a role for CTGF in increased ECM production, this finding may indicate that a single functional CTGF allele can suffice to mediate the diabetes-associated ECM increase. As such, further CTGF inhibition by antibodies or oligodeoxynucleotides might be needed in order to decrease CTGF availability to levels below those in our $CTGF^{+/-}$ mice, which remained within the normal range [12, 32-34]. Furthermore, none of these previous studies has specifically addressed dose-response issues, podocyte involvement, or GBM biology. Thus, although CTGF is an essential factor in diabetes-induced ECM production, our data indicate that a normal, physiological expression level of CTGF might be sufficient to allow DM-induced excess ECM (GBM) content.

Podocytes are also involved in GBM maintenance and remodeling by production of GBM degrading proteases, including MMPs, and their antagonists, the TIMPs [35]. MMPs are involved in degradation of ECM components, in release of growth factors from the ECM, and in modification of the cell-ECM interface [36]. For example, decreased expression of MMP-2 has been reported in a rat model of STZ-induced DM, and in glomeruli of diabetic patients [4, 37], while high glucose concentration was reported to increase MMP-9 and not MMP-2 activity in cultured podocytes [30]. Expression of TIMP-1 is strongly increased in kidneys of STZ-DM rats [37, 38]. McLennan et al. [19], reported that stimulation of human mesangial cells with either

high glucose or rhCTGF induced increased mRNA expression of MMP-2, as well as TIMP-1, which resulted in a net decreased matrix degrading capacity while, *in vivo*, more intensive insulin treatment of diabetic rats resulted in a decrease of CTGF-, and TIMP-1 expression, which led to restoration of renal cortical proteolytic activity on radiolabeled biosynthetic matrix towards normal levels, a result consistent with the present results for GBM thickness in diabetic mice [19]. Downregulation of CTGF with consequent lower TIMP expression and restoration of MMP activity has been achieved also by RAAS blockade, and by fluvastatin [39, 40]. However, not all studies have shown a decrease in MMP-2 activity caused by CTGF over-expression. For example, in cultured vascular smooth muscle cells, over-expression of CTGF or addition of CTGF to the medium resulted in increased MMP-2 activity [31].

In the present study, in situ zymography and measurements in kidney homogenates showed a (40%) decrease of gelatinase activity in diabetic $CTGF^{+/+}$ mice, but preserved (normal) gelatinase activity in diabetic $CTGF^{+/-}$ mice. The mRNA expression of MMP-2 tended to be increased more strongly in $CTGF^{+/-}$ mice compared to diabetic $CTGF^{+/+}$ mice, while the expression of MMP-9 and TIMP-1 was increased to a similar extent in diabetic $CTGF^{+/+}$, and diabetic $CTGF^{+/-}$ mice. Previously, increased MMP-2 mRNA expression in combination with approximately 30% decrease in total matrix degradative activity including 40% decrease of MMP-2 activity in diabetic kidneys has been reported also by Mc Lennan et al [41]. Together, these observations point to reduced MMP-2 activity as a possible explanation for CTGF-dependent structural and functional alterations in DN. It has been pointed out that in DN, severity of proteinuria correlates with GBM thickening [1], but it remains to be established how GBM thickening leads to glomerular dysfunction . In a more general sense, what determines the integrity of the glomerular filter has not been resolved completely, although it is evident that GBM- as well as podocyte-characteristics, and their interaction, are critically involved [42]. It seems likely that thickness of the GBM might in itself be less important than concomitant qualitative changes in its composition or associated changes in podocyte function. To resolve the possible relation between the impact of CTGF level and MMP activity on GBM thickening and albuminuria in DN, more extensive analyses will be needed.

We conclude that, in our mouse model of type 1 diabetes, increased expression of CTGF in renal cortex, and especially in glomerular podocytes, is a critical determinant of albuminuria and GBM thickening, which is associated with reduced MMP activity.

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Chapter 7

Connective Tissue Growth Factor is necessary for retinal capillary basement membrane thickening in diabetic mice

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Submitted

ABSTRACT

OBJECTIVE- Experimental prevention of basement membrane (BM) thickening of retinal capillaries ameliorates early vascular changes due to diabetes. Connective tissue growth factor (CTGF) is upregulated early in diabetes in the human retina and is a potent inducer of expression of BM components such as fibronectin and collagen type IV. We hypothesize that CTGF is causally involved in diabetes-induced BM thickening of retinal capillaries.

METHODS- In order to test this hypothesis, we compared the effect of diabetes on retinal capillary BM thickness between wild type mice and mice lacking one functional CTGF allele ($CTGF^{+/-}$). Differences in BM thickness were calculated by quantitative analysis of electron microscopical images of transversal sectioned capillaries in and around the inner nuclear layer of the retina.

RESULTS- Diabetes-induced thickening of the BM of retinal capillaries was significantly lower in mice lacking one CTGF allele.

CONCLUSIONS- Our study shows that CTGF expression is necessary for diabetes-induced BM thickening and suggests that reduction of CTGF levels may be protective against the development of diabetic retinopathy.

INTRODUCTION

Diabetic retinopathy (DR) is the leading cause of blindness in the working-age population [1]. Vascular basement membrane (BM) thickening is the most prominent and characteristic feature of early diabetic microangiopathy [2,3]. BM thickening results from increased synthesis and/or decreased breakdown of its macromolecular components such as collagen type IV, fibronectin and laminin [2,4,5].

Experimental prevention of BM thickening ameliorated early retinal vascular changes due to diabetes [6,7]. In galactose-fed rats, a model for type 2 diabetes, downregulation of fibronectin synthesis partly prevented retinal BM thickening but also reduced pericyte and endothelial cell loss [6]. Combined downregulation of the mRNA levels of the extracellular matrix components fibronectin, collagen type IV and laminin not only prevented the increase in their protein levels but also reduced vascular leakage in the retinas of rats with streptozotocin (STZ)-induced diabetes [7]. These findings suggest that BM thickening is not just an epiphenomenon of the diabetic state, but may be instrumental in the further development of sight-threatening DR. Modulation of BM thickening in man may therefore have a preventive effect on the development of DR.

Connective tissue growth factor (CTGF), a potent pro-fibrotic factor induces production of collagen, fibronectin and tissue inhibitors of metalloproteases (TIMPs) under diabetic conditions *in vitro*[8-13]. CTGF expression in the retina was upregulated in rats with STZ-induced diabetes [14] as well as in mice repeatedly infused with advanced glycation end products (AGEs) (Hughes et al., submitted). CTGF is expressed in vascular cells in the retina of diabetic humans with early diabetic microangiopathy [15].

Based on these findings, we hypothesize that CTGF plays a role in the early pathogenesis of DR by inducing capillary BM thickening and that reduction of CTGF levels is protective against diabetes-induced BM thickening. In order to test this hypothesis, we compared the effects of diabetes on retinal capillary BM thickness in wild type mice and mice lacking one functional CTGF allele.

MATERIAL AND METHODS

Genetically-modified mice

Animal experiments were performed in compliance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research. Male BalbC/129sv $CTGF^{+/-}$ mice [16] were crossbred with $CTGF^{+/+}$ female C57Bl/6J mice (Harlan, Horst, The Netherlands). The females of the F1 offspring ($CTGF^{+/-}$ and $CTGF^{+/+}$ mice) were used for the present study. The mice were genotyped and divided into 4 groups: control $CTGF^{+/+}$, control $CTGF^{+/-}$, diabetic $CTGF^{+/+}$ and diabetic $CTGF^{+/-}$. Diabetes was induced at 16 weeks of age, by means of a single i.p. injection of streptozotocin (STZ; Sigma, St. Louis, MO, USA), 200 mg/kg dissolved in 100 mM sodium citrate buffer (pH 4.6). Control animals were injected with sodium citrate buffer alone. All animals were housed in a room with constant temperature and a 12 hr light/12 hr dark cycle and were allowed standard pellet laboratory chow and water ad libitum. Induction of diabetes was determined at 3 days after injection by measurement of blood glucose levels (Medisense Precision Xtra; Abbott, Bedford, IN, USA). Slow release insulin pellets (Linshin, Scarborough, ON, Canada) were used in diabetic mice to stabilize the condition of the animals for at least 17 weeks.

As the principal aim of this experiment was to investigate the role of CTGF in diabetes-induced nephropathy, urine samples were taken at 2, 4, 6 and 9 weeks after induction of diabetes. Because albuminuria, the main characteristic of nephropathy, was still absent at 9 weeks of diabetes, unilateral nephrectomy was performed on all animals to accelerate the development of nephropathy. Urine samples were taken 2, 4 and 8 weeks after nephrectomy (i.e. at 11, 13 and 17 weeks after induction of diabetes). At 17 weeks of diabetes, the animals were killed.

To investigate the effect of the nephrectomy on BM thickness in capillaries of the mouse retina also STZ-induced diabetic $CTGF^{+/+}$ mice without nephrectomy were added to the study. Blood was collected using EDTA as anticoagulant and plasma was stored at -70°C until use. The eyes were enucleated and snap frozen in liquid nitrogen and stored until analysis.

Measurements of HbA_{1c}

HbA_{1c} was determined by means of an immunochemical method (Tina-quant; Roche/Hitache, Mannheim, Germany).

CTGF ELISA

CTGF levels in plasma and urine were determined by means of sandwich ELISA using 2 distinct specific antibodies both directed against the CTGF protein (FibroGen, South San Francisco, CA, USA) as was described previously [17].

Microtiter plates were coated overnight at 4°C with 50 µl capture goat-anti-CTGF-N polyclonal antibody (10 µg/ml) in coating buffer (0.05 M sodium bicarbonate, pH 9.6). The following day, the plates were blocked with 1% BSA in PBS for 2 hours at room temperature and washed with wash buffer (PBS/0.05% Tween 20). Plasma samples were diluted 1:10 and urine samples 1:2 in assay buffer (50 mM Tris, pH 8.0, 0.1% BSA, 50 mg/l sodium heparin, 0.1% Triton X-100) and 100 µl diluted sample was added to each well. Purified recombinant human CTGF (FibroGen) was used as a standard. Plates were incubated for 2 hours at room temperature, washed and incubated with 100 µl monoclonal anti-CTGF-N antibody (FG- 3019; FibroGen; 4 µg/ml in assay buffer). Plates were washed and incubated with 100 µl/well goat anti-human IgG alkaline phosphatase (Sigma; 1:2000 diluted in assay buffer). Plates were washed again and 100 µl of substrate solution containing p-nitrophenyl phosphate (Sigma) was added to each well. After 20-30 minutes incubation in the dark, absorbance was read at 405 nm on a Biorad microplate reader. This assay detects full-length as well as N-terminal fragments of CTGF. To avoid confusion due to differences in the molecular weight of full-length and different fragments of CTGF, all levels were expressed as pmol/l.

Quantitative RT-PCR

The mRNA levels of CTGF and TGF-β1 in total renal cortex were quantified by real-time quantitative PCR. For this purpose, RNA was isolated from frozen sections of renal cortex using the RNeasy minikit (Qiagen, Hilden, Germany). cDNA synthesis was performed on 3 µg of RNA using oligo-$dT_{12\text{-}18}$ and Superscript reverse transcriptase (Invitrogen, Carisbad, CA, USA).

Quantitative RT-PCR was performed using a SYBR Green kit (Applied Biosystems, Foster City, CA, USA) and analyzed on an ABI PRISM 7700 Sequence Detector System (Applied Biosystems). Genes and primer sequences are presented in Table 1. The thermal

Table 1: Forward and reverse sequences of the primers used in the present study.

CTGF	
Forward:	5'-CACAGAGTGGAGCGCCTGTTC-3'
Reverse:	5'-GATGCACTTTTTGCCCTTCTTAATG-3'
TGF-β1	
Forward:	5'-GCAACATGTGGAACTCTACCAGA-3'
Reverse:	5'-GACGTCAAAAGACAGCCACTCA-3'
TBP	
Forward:	5'-CAGGAGCCAAGAGTGAAGAAC-3'
Reverse:	5'-GGAAATAATTCTGGCTCATAGCTACT-3

CTGF, connective tissue growth factor; TGF, transforming growth factor; TBP, TATA box-binding protein.

cycling comprised a denaturation step at 95 °C for 10 minutes followed by 45 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. To confirm that only one single PCR product was detected, the PCR products were analyzed using a heat dissociation protocol. Quantitative values were obtained from the threshold PCR cycle number, at which the increase in signal associated with an exponential growth of PCR product can be detected. The relative mRNA levels in each sample were normalized for the TATA box-binding protein (TBP) contents.

Tissue samples and processing of the mouse eyes

In total, 30 eyes of 15 mice were collected from the experiment. The distribution of the mice within the experimental groups was as follows: 2 wild type controls (C+/+), 5 CTGF heterozygous controls (C+/-), 5 wild types with diabetes (DM+/+) and 3 CTGF-heterozygous with diabetes (DM+/-). To investigate whether there was a possible effect of nephrectomy on BM thickness, control eyes of 2 C+/+ mice without nephrectomy were collected and analyzed in parallel.

Electron microscopy

One eye of each mouse was cut in 2 pieces and defrosted and fixed in Peter's fixative containing 1% paraformaldehyde (Sigma, Zwijndrecht, the Netherlands) and 1.25% glutaraldehyde (Sigma) buffered in 0.1 M sodium cacodylate (Sigma), pH 7.4, for 2 h. Retinas were extracted and washed in Peter's buffer (sodium cacodylate 0.1 M, pH 7.4). The retinas were postfixed in osmium tetroxide (Merck, Haarlem, the Netherlands) supplemented with 1% potassium ferricyanide in Peter's buffer for 1 h, subsequently dehydrated and embedded in Epoxy resin (Epon 8.12, Merck). Ultrathin sections were cut from the resin block on a Reichert-Young ultramicrotome (Leica Microsystems, Nussloch, Germany) equipped with a Diatome diamond knife. Sections were mounted on 200-mesh naked copper grids.
Of every retina, 20-30 images of transversal sectioned capillaries in and around the inner nuclear layer were photographed at x 40.000 initial magnification using a EM 201 electron microscope (Philips, Eindhoven, the Netherlands), with a final on screen resolution of 3.7 nm. The images were analyzed with a validated software package (Cap-Image, Dr. H. Zeintl Engineering, Heidelberg, Germany).

Basement membrane measurements

Ten images of capillaries were randomly selected from the 20-30 images per retina and measurements were made blindly. Images were projected against "a sun" with 20 equally spaced radians as described by Cuthbertson and Mandel [18]. Where the radial lines intersected the BM, BM thickness was measured. Both "inner" and "outer" BMs were measured. These were defined according to Fischer and Gärtner [19], as "inner"

BM (IBM) when positioned between endothelial cell and pericyte and as "outer" BM (OBM) when positioned between either endothelial cell or pericyte and the surrounding glial tissue, respectively. Readings were taken only when the cell membranes bordering the BM were clearly visible as sharp black lines. Capillaries were excluded from the study when less than 12 measurements could be made.
In addition, a second approach was followed to blindly measure the BM thickness in the same 10 randomly-selected images of capillaries as described above. In this case, the BM of each capillary was divided into 3 regions: BM of the endothelial cell (EBM), BM of the pericyte (PBM) and BM of both endothelial cell and pericyte (BBM) (Figure 1). The images were rastered and in each raster, BM thickness was measured twice by drawing computer-lines. Lines were drawn only when the cell membranes bordering the BM were clearly visible as sharp black lines. In this way, 10-20 measurements per region (30-60 per capillary) were made.

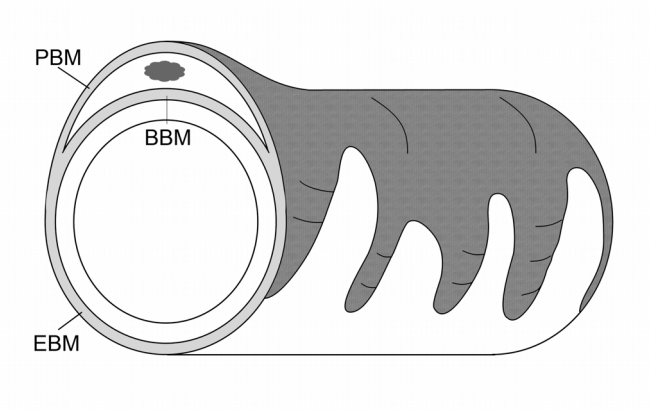

Figure 1: Schematic representation of a retinal capillary with an endothelial cell and pericyte. The BM in the endothelial cell region is marked EBM. The BM in the pericyte region is marked PBM and the basement membrane between both cell types is marked BBM.

Statistical analysis

Mean "inner" BM thickness and mean "outer" BM thickness was calculated per capillary (first method). Differences between "inner" and "outer" BM thickness among groups were analyzed using ANOVA with repeated measurements option (SPSS 12.02; SPSS Inc., Chicago, IL, USA). In this way, individual mean values per capillary were used during analysis instead of mean values per mouse.
With respect to the second method, all data were transformed into rank cases because of a skewed distribution. The same ANOVA with repeated measurements option was used to calculate differences between capillary regions among the experimental groups. P-values <0.05 were considered statistically significant.

RESULTS

Effect of nephrectomy on BM thickness in retinal capillaries of the mouse

Nephrectomy did not affect the BM thickness in capillaries of the mouse retina (mean OBM of wild type ($CTGF^{+/+}$) control mice *without* nephrectomy was 0.148 µm and mean OBM of $CTGF^{+/+}$ control mice *with* nephrectomy was 0.149 µm; P=0.875). Therefore, for further analyses, the 2 control groups (with and without nephrectomy) were taken together.

Induction of diabetes

Development of diabetes was established by measurement of blood glucose and HbA_{1c} levels (Table 2). The difference in blood glucose and HbA_{1c} levels between control animals and diabetic animals was highly significant ($p<0.001$), whereas the blood glucose and HbA_{1c} levels did not differ between the $CTGF^{+/+}$ and $CTGF^{+/-}$ control mice and between diabetic $CTGF^{+/+}$ and diabetic $CTGF^{+/-}$ mice.

Table 2: Blood glucose and HbA1c levels (mean ± SD) of diabetic and control $CTGF^{+/+}$ and $CTGF^{+/-}$ mice.

Parameters	Control		Diabetic	
	CTGF+/+	CTGF+/-	CTGF+/+	CTGF+/-
n	4	5	5	3
Blood glucose (mmol/l)	8.5 ± 0.4	7.6 ± 0.8	> 25.0	> 25.0
HbA_{1C} (%)	4.6 ± 0.1	4.8 ± 0.2	9.3 ± 1.5	8.6 ± 1.4

CTGF gene dose effect

A gene dose effect of the lack of one functional allele coding for CTGF was established in plasma and urine at the protein level. In control mice, CTGF levels in plasma and urine were significant lower (approximately 50%) in control $CTGF^{+/-}$ mice as compared to control $CTGF^{+/+}$ mice. In diabetic mice, CTGF levels in plasma and urine in diabetic $CTGF^{+/-}$ mice were lower compared to diabetic $CTGF^{+/+}$ mice, albeit not significantly in urine (Figure 2 A and B).

CTGF mRNA levels of the total renal cortex as measured by means of PCR were not significantly different in control $CTGF^{+/+}$ and $CTGF^{+/-}$ mice. However, in diabetic animals, there was a 3-fold increase in CTGF mRNA expression in total renal cortex of diabetic $CTGF^{+/+}$ mice as compared to control $CTGF^{+/+}$ mice, whereas the increase in CTGF mRNA levels in diabetic $CTGF^{+/-}$ mice was only 1.5 fold as compared to $CTGF^{+/+}$ control mice (Figure 2C). TGF-β1 mRNA levels in the total renal cortex, measured by means of PCR, were affected only by induction of diabetes, and not by CTGF genotype (Figure 2).

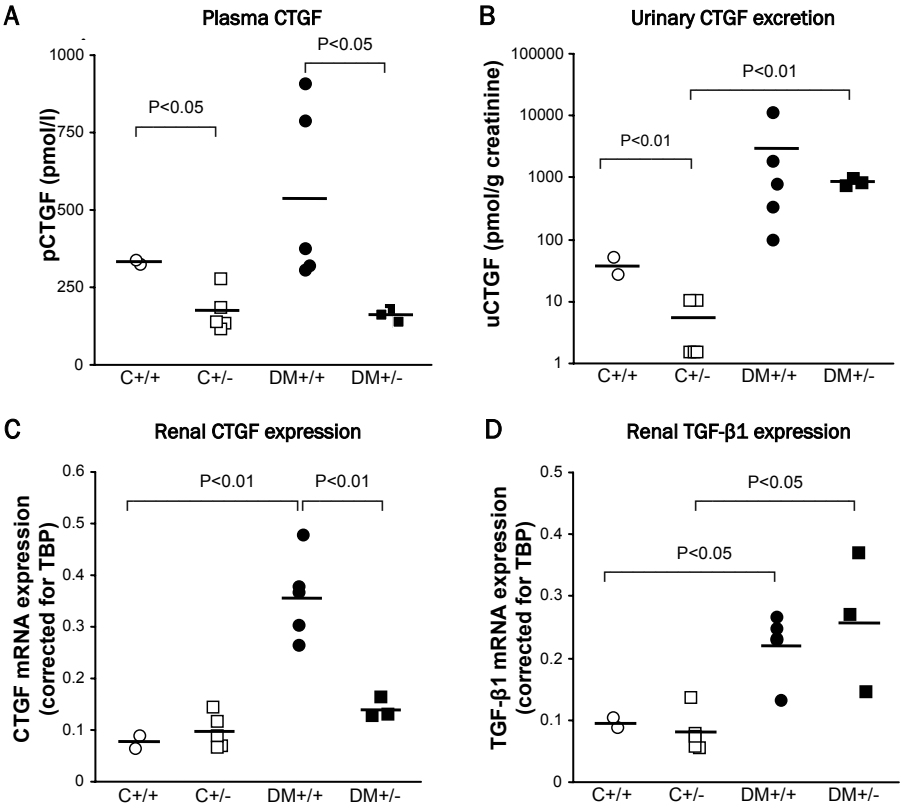

Figure 2: CTGF protein levels in A: plasma and B: urine of control and diabetic CTGF$^{+/+}$and CTGF$^{+/-}$ mice. Note the significant lower CTGF protein levels in CTGF$^{+/-}$ mice (C+/- and DM+/-) as compared to CTGF$^{+/+}$ mice (C+/+ and DM+/+). C: The induction of CTGF mRNA levels in the renal cortex in diabetes was significantly lower in CTGF$^{+/-}$ mice (DM+/-) as compared to CTGF$^{+/+}$ mice (DM+/+). D: No effect of the genotype was noted with respect to TGFβ-1 mRNA levels in the renal cortex. The significant induction of TGFβ-1 mRNA levels due to diabetes was similar in CTGF+/- mice (DM+/-) as compared to CTGF+/+ mice (DM+/+).

Effect of diabetes on BM thickness in wild type mice

The thickness of the OBM was significantly increased in diabetic CTGF$^{+/+}$ (wild type) mice compared to CTGF$^{+/+}$ controls (P=0.03) (measured according to Cuthbertson and Mandel [18] (Figure 3). The thickness of the IBM was not affected by diabetes in CTGF$^{+/+}$ mice (Table 3). Regional BM analysis demonstrated that the BM in the endothelial cell region (EBM) and the BM in the pericyte region (PBM) were thicker in diabetes. However, only the increase in EBM thickness was significant (P=0.03). The BM in between (BBM) was not affected by diabetes in wild type mice (Table 3; Figure 3).

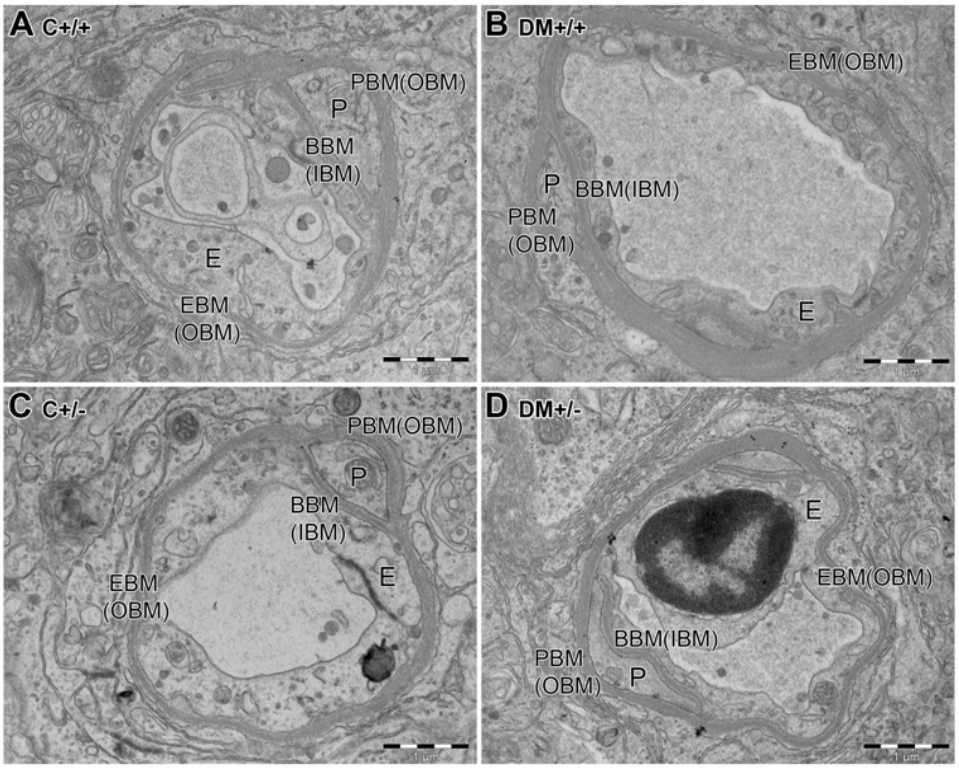

Figure 3: Examples of retinal capillaries analyzed in the present study. The basement membranes (BMs) are identified as "outer" BM (OBM) and "inner" BM (IBM) in the analysis according to Fischer and Gärtner 19. In the analysis of the BM regions, the BM was divided into three regions: endothelial BM (EBM), pericyte BM (PMB) and the BM in between both cell types (BBM). Note that the IBM and BBM are the same region as well as OBM end EBM plus PBM. Note the diabetes-induced increase in BM thickening in the diabetic $CTGF^{+/+}$ (wild type) animals (DM+/+) (B) when compared to the $CTGF^{+/+}$ (wild type) control mice (C+/+) (A) and the absence of this effect in the diabetic $CTGF^{+/-}$ mice (DM+/-) (D) when compared to the non-diabetic $CTGF^{+/-}$ control mice (C+/-) (C).

Effect of CTGF genotype on BM thickness

In contrast to the wild type animals, diabetes did not induce an increase in BM thickness in diabetic $CTGF^{+/-}$ (CTGF heterozygous) mice (Table 3).

BM thickness measurements of control $CTGF^{+/+}$ and control $CTGF^{+/-}$ mice without diabetes were not significant different in all cases and in all regions. However, control $CTGF^{+/-}$ mice tended to show a somewhat thicker BM than control $CTGF^{+/+}$ mice.

Table 3: BM thicknesses of retinal capillaries in control and diabetic $CTGF^{+/+}$ and $CTGF^{+/-}$ mice

BM thickness	Wild type mice ($CTGF^{+/+}$)		CTGF heterozygous mice ($CTGF^{+/-}$)	
	$CTGF^{+/+}$ control (n=4)	$CTGF^{+/+}$ DM (n=5)	$CTGF^{+/-}$ control (n=5)	$CTGF^{+/-}$ DM (n=3)
OBM	**0.148** ± 0.022	**0.165** ± 0.027	0.155 ± 0.029	0.160 ± 0.030
IBM	0.120 ± 0.017	0.127 ± 0.020	0.126 ± 0.022	0.124 ± 0.030
Endothelial region (EBM)	**0.142** (0.090-0.233)	**0.157** (0.097-0.235)	0.151 (0.097-0.247)	0.144 (0.094-0.233)
Pericyte region (PBM)	0.165 (0.127-0.251)	0.182 (0.121-0.330)	0.167 (0.081-0.269)	0.174 (0.108-0.269)
Region between (BBM, IBM)	0.115 (0.079-0.156)	0.132 (0.099-0.216)	0.129 (0.047-0.239)	0.119 (0.078-0.269)

Thickness of outer BM (OBM) and inner BM (IBM) are expressed as mean ± standard deviation. Thickness of BM regions is expressed as median with range. The mean thickness of the OBM is significant different between $CTGF^{+/+}$ control and diabetic mice as is the median thickness in the EBM region in these mice (values in bold; $P \leq 0.05$).

DISCUSSION

Our study shows that mice lacking one allele of the CTGF gene fail to develop BM thickening after 4 months of diabetes. Our findings indicate that CTGF is necessary for BM thickening and identify CTGF as a possible therapeutic target to prevent this early change in the retina caused by diabetes. That this may be a clinically-relevant approach is indicated by recent studies which have shown that prevention of BM thickening can ameliorate the subsequent development of acellular capillaries in rodent models of diabetes [6,7].

We detected a significant increase in "outer" BM thickness in diabetic $CTGF^{+/+}$ mice when compared to non-diabetic $CTGF^{+/+}$ control mice. The second independent regional analysis of the BM showed similar results. The observed thickening of the "outer" BM and not the "inner" BM due to diabetes in mice has been noted previously [19]. Remarkably, the effect of diabetes on BM thickness was absent in the diabetic mice lacking one CTGF allele (diabetic $CTGF^{+/-}$).

The diabetic state of these diabetic $CTGF^{+/-}$ mice was comparable with that of diabetic wild type ($CTGF^{+/+}$) mice, as was indicated by similar blood glucose and HbA_{1c} levels. However, there was a clear genotype effect on CTGF expression in the CTGF heterozygous mice. The lack of one functional allele coding for CTGF was associated with approximately 50% lower CTGF protein expression levels in plasma and urine of control mice. Although the CTGF mRNA levels were not different in non-diabetic wild type and CTGF heterozygous control mice, a genotype effect on CTGF mRNA levels was evident in diabetic mice. Renal CTGF mRNA levels in diabetic $CTGF^{+/-}$ mice were only 50% of those in diabetic $CTGF^{+/+}$ mice. Local ocular CTGF mRNA and protein levels were not analyzed in the present study. However, the above mentioned renal and systemic levels of CTGF suggest that the lower levels of circulating and possibly also retinal CTGF protein in our CTGF heterozygous mice may have prevented diabetes-induced BM thickening in retinal capillaries.

This finding is in line with the known function of CTGF as potent inducer of ECM synthesis by means of increasing production of collagen, fibronectin and TIMPs, including under diabetic conditions in cultured cells [8,9,11,12,20-24]. In general, CTGF exerts these effects on cell proliferation, differentiation and production of collagen and fibronectin as downstream mediator of TGF-β [25-27]. However, CTGF is also independently of TGF-β capable of exerting these functions [28,29].

A causal role of TGF-β in the induction of capillary BM thickening has already been demonstrated in brain capillaries in the mouse, as well as in diabetes-induced BM thickening of glomerular capillaries in the kidney [30-32]. However, inhibition of TGF-β to prevent BM thickening and thereby ameliorating early retinal vascular changes due to diabetes may have severe adverse effects due to its important physiological functions in tumor suppression and dampening of inflammation [33]. Because CTGF

can also be upregulated independently of TGF-β by high glucose levels, through activation of the renin-angiotensin system [34-36], by AGEs [24] and by vascular endothelial growth factor (unpublished results), CTGF could be a more attractive therapeutic target to attenuate diabetes-induced BM thickening of retinal capillaries. Further studies are needed to determine the exact role of CTGF activity in the retina.

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Chapter 8

Possible mechanisms of CTGF action in the pathogenesis of diabetes-induced GBM thickening

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Manuscript in preparation

INTRODUCTION

Thickening of the glomerular basement membrane (GBM) and (micro-) albuminuria are the main early characteristics of diabetic nephropathy (DN). Growth factors are known to play an important role in the pathogenesis of DN [1]. Especially TGF-β has been described to be critically involved in this process. However, since TGF-β is also involved in important anti-proliferative and anti-inflammatory processes, anti-TGF-β therapy as a strategy for treatment of DN might be accompanied by serious side effects [2]. In this thesis we present studies on the role of connective tissue growth factor (CTGF), a downstream mediator of the pro-fibrotic action of TGF-β, in the development and progression of DN, and the potential of anti-CTGF therapy as treatment for DN.

One of the main observations of our studies was that reduced CTGF expression in diabetic CTGF heterozygous ($CTGF^{+/-}$) mice compared to diabetic wild type ($CTGF^{+/+}$) mice resulted in complete prevention of GBM thickening and was accompanied by a significant reduction of albuminuria (chapter 6). A similar effect has been described in a preliminary report by Usinger et al, who treated type 2 diabetic db/db mice with CTGF-neutralizing antibodies [3].

Diabetes induced GBM thickening can be caused by increased synthesis or decreased breakdown of GBM proteins, e.g. collagen IV, but also by alterations in its architecture and composition [4, 5]. As for architecture and composition, increased pore size and decreased charge selectivity of the GBM have been reported [6, 7]. In diabetic patients with normoalbuminuria the pores in the GBM were more enlarged and irregular in shape compared to control subjects and these ultrastructural changes became even more obvious in patients with microalbuminuria [7]. In diabetic rats, the size of the pores in the GBM was increased while the number of anionic sites in the GBM was significantly decreased already 2 weeks after induction of diabetes. In addition, albuminuria was significantly increased after 4 weeks [6]. Prevention of the thickening and loss of anionic charge of the GBM by treatment with aminoguanidine, an inhibitor of AGE formation, was associated with a decrease in albuminuria [8]. Besides the GBM, also the slit diaphragm between the foot processes of the podocytes has been implicated as filtration barrier [9]. There is still no consensus as to whether the GBM or the slit diaphragm constitutes the primary glomerular filtration barrier [10]. However, in a recent study in mice with a severely disorganized GBM it was shown that proteinuria precedes podocyte abnormalities [11]. This underscores the importance of the GBM as a main determinant of integrity of the glomerular filtration barrier.

GBM COMPONENTS AND THEIR REGULATION

The GBM is a fibrillar meshwork of extracellular matrix proteins including specific isoforms of laminin, collagen IV, entactin and proteoglycans [12]. It has been shown that diabetic conditions increase the expression of collagen IV [13], fibronectin [14] and several other matrix components in podocytes and other glomerular cells, thus contributing to thickening of the GBM in DN [5]. Both TGF-β and CTGF are involved in diabetes-induced increase of the expression of GBM components [5, 15].

We have determined the renal cortical mRNA and protein expression of several GBM proteins in both $CTGF^{+/+}$ and $CTGF^{+/-}$ diabetic mice (chapter 6). Despite an increased expression of several obvious GBM proteins in both groups of diabetic mice, we did not observe a relation between the expression level of these proteins and GBM thickening or CTGF overexpression. This suggested that, instead of increased expression of other GBM components, architectural changes or impairment of physiological GBM turnover/degradation might be responsible for the observed CTGF-dependent GBM thickening in diabetic mice. Therefore, we also investigated whether CTGF influences the GBM thickness through impairment of its proteolytic degradation by matrix metalloproteinases.

REDUCED ACTIVITY OF MATRIX METALLOPROTEINASES IN DIABETIC MICE

Matrix metalloproteinases (MMPs) are zinc-dependent enzymes that are importantly involved in ECM turnover under physiological and pathological conditions [16]. MMP-2 and -9, also known as gelatinase A and B, are involved in proteolytic degradation of collagen IV, the main component of basement membranes, other collagens, elastin, fibronectin and, laminin. In addition, they are the only MMPs able to degrade gelatin [17]. This gelatinase activity can be used experimentally as a tool to measure specifically the matrix degrading activity of MMP-2 and -9.

The significant increase in GBM thickness in diabetic wild type $CTGF^{+/+}$ mice, as described in chapter 6, was accompanied by a 40% decrease in gelatinase activity. A role of CTGF in these changes was suggested by our observation that in diabetic $CTGF^{+/-}$ mice, who had normal instead of increased GBM thickness, gelatinase activity was preserved and not different from non-diabetic controls. A significant decrease in collagen IV degrading activity of MMPs in combination with GBM thickening in STZ-diabetic rats has been reported previously by McLennan et al. [18], who observed that the decrease in renal matrix degrading activity in diabetes resulted from decreased activity of both MMP-2 and MMP-9, as was evidenced by gelatine zymography.

Despite the observed reduction in MMP activity, the renal mRNA expression of MMP-2 was significantly increased in our diabetic mice. In correspondence with our results, McLennan observed an increase in MMP-2 mRNA expression in diabetic rats [18]. In contrast, however, in the latter study the mRNA expression of MMP-9 was significantly

impaired, while our diabetic mice showed a tendency towards increased MMP-9 mRNA expression. Also in vitro studies have shown differential effects of high glucose on MMP-2 and MMP-9 mRNA expression. High glucose increased the mRNA expression of MMP-2 in human mesangial cells *in vitro* [19]. On the other hand, podocytes cultured in high glucose had increased MMP-9 expression, while MMP-2 expression was unaffected [20]. Despite these apparently dissimilar observations on diabetes-related mRNA and protein regulation of these proteases, the renal matrix degrading capacity for gelatin and collagen IV appears to be consistently decreased under (long-standing) diabetic conditions [18-21]. It is important to note that the activity of MMPs is not only regulated at the transcriptional and translational level, but that also secretion, (proteolytic) activation, and inhibition by specific inhibitors are important (co-) determinants [16].

REGULATION OF MMP ACTIVITY: GENERAL ASPECTS

MMP activity is regulated at the level of gene transcription, translation, secretion, proteolytic activation, and by tissue inhibitors of metalloproteinases (TIMPs). MMPs are secreted into pericellular compartments as latent zymogens which require proteolytic processing of their N-terminal region to obtain catalytic activity. Pro-MMP-2 and pro-MMP-9 can be activated by many natural activators or special chemical substances. Both in physiological and pathological conditions, the plasminogen/plasmin system is one of the most common activators and modulators of this process. Pro-MMP-2 is activated on the plasma membrane by MT1-MMP and TIMP-2 (!) [22], and by plasmin [23], while pro-MMP-9 is activated by proteolytically active MMP-2 or MMP-3 [17, 23].
Plasminogen activator inhibitor-1 (PAI-1) inhibits the formation of plasmin out of plasminogen by uPA (urokinase-type plasminogen activator) or tPA (tissue-type plasminogen activator) [24]. In mesangial cells cultured in high glucose, the expression of PAI-1 is increased and plasmin activity is decreased [25, 26]. In addition, renal mRNA expression of PAI-1 was increased in kidneys of experimental diabetic rats [25, 27] and circulating PAI-1 levels were higher in diabetic patients with diabetic nephropathy than in patients with non-diabetic nephropathy [28]. Thus, by means of increased PAI-1 expression, diabetes can impair the activation of MMPs. CTGF has been implicated in TGF-β induced expression of PAI-1 [29]. In this way, upregulation of CTGF might be an important determinant of the reduced activation of MMP-2 and MMP-9 via PAI-1 in diabetes.
Still other factors are involved in the regulation of MMP activity. For example, the expression of MT-1 MMP is decreased in mesangial cells upon culture in high glucose medium [30]. Moreover, MMPs can be inhibited by their specific inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). To date, four TIMPs (TIMP-1 to TIMP-4) have been identified in vertebrates. TIMP-1, TIMP-2, and TIMP-4 are soluble proteins

whereas TIMP-3 is anchored in the extracellular matrix [31]. TIMPs inhibit MMPs in a 1 : 1 stoichiometry and in a reversible manner. The four TIMPs have different affinity for the different MMPs. TIMP-2 is able to form complexes with pro-MMP-2 and TIMP-1 binds pro-MMP-9 [32]. In contrast, both TIMP-1 and TIMP-2 are able to bind active MMP-2, although in a different manner [32]. TIMP-2 is involved in pro-MMP-2 activation as a co-factor of MT-1 MMP, but an excess of TIMP-2 inhibited pro-MMP-2 activation [33, 34].

TIMP expression in tissues is tightly regulated to maintain appropriate levels of proteolytic activity. Dysregulation of TIMPs or MMP activity leads to of either too high MMP activity with exaggerated extracellular matrix turnover and remodeling via impaired repair and scar formation or too low MMP activity, causing extracellular matrix accumulation and fibrosis. The mRNA expression of TIMPs is increased in kidneys of mice and rats with experimentally induced diabetes ([18, 21] and chapter 6). CTGF can mediate inhibitory effects of high glucose on matrix degradation of MMPs by increasing TIMP-1 mRNA and protein expression [19]. Thus, induction of TIMPs by CTGF might be responsible for decreased MMP activity in diabetes. In chapter 6 we did observe a significant increase of TIMP-1 mRNA expression in diabetic as compared to control mice, but no difference between diabetic $CTGF^{+/-}$ mice and diabetic $CTGF^{+/+}$ mice. However, a definitive conclusion was precluded by relatively small group size and a relatively large variation in TIMP-1 expression.

One of the pathways by which MMPs are degraded involves the low density lipoprotein receptor-related protein (LRP) [35] which is also known to interact with CTGF, as will be described in more detail below.

ROLE OF CTGF IN REGULATION OF MMP ACTIVITY

In chapter 6 we show that the diabetes-induced decrease of MMP activity was completely prevented in diabetic $CTGF^{+/-}$ mice. In these diabetic animals, the lack of one functional CTGF allele was accompanied by an only marginal diabetes-induced increase of CTGF and preserved MMP activity, in contrast to the marked increase of CTGF and decrease of MMP activity in diabetic wild type littermates. It thus appears that CTGF overexpression is responsible for impaired MMP activity under diabetic conditions. There are several ways how CTGF might influence MMP activity (figure 1). Possible mechanisms of CTGF action can be categorized in: 1) modulation of growth factor signaling by physical interaction of CTGF and 2) induction of intracellular signaling by direct binding of CTGF to cell surface proteins.

PHYSICAL INTERACTION OF CTGF WITH GROWTH FACTORS

CTGF can form molecular complexes with several growth factors. By binding to these growth factors, CTGF is able to modulate their action. Here we discuss possible mechanisms by which binding of CTGF to TGF-β, BMP, VEGF and IGF can influence the activity of MMP-2 and -9.

TGF-β and BMP

Both TGF-β and BMP (bone morphogenetic protein) can influence target gene expression through receptor-mediated intracellular signaling via phosphorylation of Smad proteins. Abreu showed that, although CTGF itself can not directly bind to the TGF-β- and BMP receptors, it can influence the fibrotic response via the Smad signaling pathway by means of interactions with TGF-β and BMP. Binding of CTGF to TGF-β1 enhances the binding of TGF-β1 to the type II TGF-β receptor (TβRII) while binding of CTGF to BMP lowers the affinity of BMP(-4) for BMP receptors (BMPR) [36].

Once the type II receptor is activated by ligand-binding, the type I receptor is recruited, which subsequently recognizes and phosphorylates pathway-specific receptor-regulated Smads (R-Smads) that reside in the cell cytoplasm. The “pro”-fibrotic pathway by TGF-β involves R-Smad 2 and 3 while the “anti”-fibrotic pathway by BMP involves R-Smad 1, 5 and 8. Phosphorylation of an R-Smad releases it from the receptor complex and allows its complexation with the co-Smad (Smad 4), which is common to the TGF-β and BMP signaling pathways. The R-Smad/Smad4 complex then translocates to the nucleus where it recognizes regulatory elements in target genes with the help of associated DNA-binding cofactors [37].

Smad binding elements (SBE) are found in the promotor region of numerous TGF-β induced genes. Genes regulated via the TGF-β/Smad pathway include: CTGF [38], collagens [39, 40], MMP-2 and MMP-9 [41], PAI-1 [42], TIMP-1 [43], Smad-7 [44], α-SMA [45], and the PDGF-β chain [46].

In addition to enhancement of receptor binding by TGF-β, CTGF also enhances the signaling activity and thus the protein expression via the TGF-β1/Smad pathway. At low TGF-β1 concentrations, CTGF potentiated the phosphorylation of Smad2 induced by TGF-β1 in fetal mink lung cell cultures. At higher concentrations of TGF-β1, CTGF did not increase Smad2 phosphorylation, indicating that CTGF serves to potentiate the effects of limiting amounts of TGF-β1. However, it had no detectable effects of its own on the Smad signaling activity in these cells [36].

Abreu and co-workers [36] have shown that, by binding to BMP, CTGF inhibits signaling of BMP-4 via the BMP/Smad pathway. Little is known about BMP-4 in MMP regulation, diabetes, or renal ECM homeostasis. However, signaling of BMP-7 via the Smad/pathway was found to counteract the induction of pro-fibrotic genes via increase of the expression of Id proteins [47]. CTGF, PAI-1 and thrombospondin-1 (TSP-1) are

among the TGF-β responsive genes that are directly downregulated by BMP-7 [48]. In addition, blocking of the TGF-β-induced upregulation of PAI-1 by BMP-7 also resulted in increased expression of MMP-2 [49] and in enhanced activity of the urokinase-type plasminogen activator [50]. The latter results in enhancement of the transformation of plasminogen into plasmin [23], which in turn is involved in the activation of MMP-2 and MMP-9.

Recently, it has been shown that BMP-4 as well as BMP-6 can substitute for BMP-7 loss during kidney development, which exemplifies that different BMP family members can function interchangeably to activate essential signaling pathways for growth and morphogenesis of the kidney [51]. Although this has not been resolved experimentally, it has been speculated that, like BMP-4 also BMP-7 signaling can be inhibited by CTGF. The possible interaction of CTGF with BMP-6 and BMP-7 is currently under investigation in our lab.

In conclusion, by binding TGF-β and BMPs, CTGF is thought to modulate the equilibrium between the expression of pro-fibrotic genes via TGF-β and the expression of potentially anti-fibrotic genes via BMP. Through enhancement of the TGF-β/Smad signaling pathway, CTGF upregulates the expression of MMP-2 and -9, but also of their inhibitors, the TIMPs, and of PAI-1, which inhibits the activation of the pro-MMPs. In addition, inhibition of the BMP/Smad signaling pathway by CTGF might further reduce the activation of MMP-2 and MMP-9. Thus, disturbance of the TGF-β/BMP balance due to CTGF overexpression in diabetes might be an important cause of reduced MMP activity and (consequent) thickening of the GBM.

VEGF

Vascular endothelial growth factor (VEGF) can form complexes with CTGF through binding to its domain 3 [52]. Interaction of CTGF with VEGF has been reported to inhibit the angiogenic activity of VEGF [52]. Both CTGF and VEGF are highly expressed in podocytes of experimental diabetic animals [53, 54]. MMPs can cleave VEGF-bound CTGF resulting in release and reactivation of VEGF, although the proteolytic activity of MMP-2 and MMP-9 to CTGF-VEGF complexes is very weak [55]. Of interest, forced overexpression of MMP-9 in human breast cancer MCF-7 cells resulted in increased tumor angiogenesis, tumor growth, and VEGF/VEGF-receptor complex formation [56]. It was hypothesized that MMP-9 controls the release of VEGF from the ECM. It seems plausible that this involves also release of active VEGF from (matrix-bound) CTGF [57, 58].

Furthermore, angiogenic stimulation with VEGF has resulted in shedding of membrane vesicles containing MMP-2, MMP-9 and MT1-MMP by endothelial cells, which also plays an important role in angiogenesis by degradation of the vascular basement membrane and remodeling of the ECM in order to allow endothelial cells to migrate

and invade into the surrounding tissue [59-61]. Thus, also inhibition of VEGF by CTGF in the diabetic glomerulus might contribute to GBM thickening by decreasing the release and activation of MMPs.

IGF

Insulin-like growth factor (IGF)-I is yet another binding partner of CTGF [62, 63]. IGF-I is a mitogenic factor that promotes ECM accumulation in various cell types [64-68]. Like CTGF, IGF-I can be induced by high glucose conditions in cultured renal fibroblasts [63]. In experimental models of diabetic kidney disease, increased renal IGF-I levels were correlated with pathological changes [64].

In vitro stimulation of renal fibroblasts with CTGF and IGF-I induced collagen production, also in the absence of TGF-β signaling [63]. In addition to IGF-I, also IGF-II is involved in ECM production by cultured mesangial cells [66]. Furthermore, IGF-II has been shown to influence the biological effects of CTGF. Grotendorst and co-workers showed that IGF-II is required for cells to differentiate into myofibroblasts and to increase collagen synthesis in response to TGF-β and CTGF [69]. However, direct interaction of CTGF with IGF-II has not been shown.

IGF-I and IGF-II both signal via the IGF-I receptor (IGF-IR) [70]. The IGF-IR has been identified as a regulator of carcinoma cell invasion and MMP-2 synthesis and activity via two different pathways dependent on the ligand load. At a concentration of 10 ng/ml, which is optimal for mitogenesis, cellular proliferation, and motility [71], IGF-I activates the MMP-2 promoter and also enhances MMP-2 protein synthesis via the PI-3K/Akt pathway. Besides enhancement of MMP-2 expression and synthesis, via PI-3K/Akt was also shown to be involved in induction of MT1-MMP expression [72].

In contrast, at higher ligand concentrations, signaling of the IGF-R downregulates MMP-2 via the Raf/MEK/ERK pathway [73]. Crosstalk of this ERK pathway with the Smad signaling pathway has been reported to result in enhancement of the collagen I expression [74]. Thus, if the enhancing effect of CTGF on IGF-R signaling mimics increased ligand load, this might represent an additional pathway of CTGF-dependent impairment of MMP activity and GBM thickening in diabetes.

DIRECT BINDING OF CTGF TO CELL SURFACE RECEPTORS

CTGF has been shown to interact directly with several cell surface proteins. Interactions of CTGF have been described with the low-density lipoprotein receptor-related protein (LRP), with several integrins, and also recently with the neurothrophin receptor TrkA. Here we describe how these interactions might possibly contribute to decreased activity of MMP-2 and -9.

Binding of CTGF to LRP

It has been shown that CTGF can bind to the low-density lipoprotein receptor-related protein (LRP) [75, 76]. LRP is a heterodimeric endocytic receptor comprising a large ligand binding subunit and a non-covalently associated transmembrane partner. It can bind multiple unrelated ligands like apolipoprotein E containing lipoproteins and α_2-macroglobulin protease complexes [77]. Currently more than 30 biologically divers partners have been identified that bind to LRP [76, 77]. Binding of CTGF to LRP resulted in internalization and degradation of CTGF [76, 78]. It is conceivable that binding of CTGF to LRP in turn affects the binding of other molecules to LRP. In addition, CTGF has also been described to signal via LRP, thus potentiating TGF-β induced myofibroblast differentiation [78].

LRP has also been described to be involved in regulation of MMP activity. LRP can bind complexes of pro-MMP-2 with TIMP-2 or thrombospondin-2 as well as complexes of pro-MMP-9 with TIMP-1. Binding of these MMP-complexes promotes their accelerated endocytic uptake and lysosomal degradation thus reducing the local gelatinase activity [35].

In addition to internalization of CTGF and MMPs, LRP is a co-receptor to the urokinase receptor (uPAR) and thus involved in the metabolism of PAI-1 [79]. Through the combined action of uPAR and LRP, the uPA/PAI-1 complex is internalized and degraded in lysosomes, whereas the uPAR is recycled back to the cell surface [80, 81].

Reduction of LRP expression has been suggested to represent a powerful mechanism for malignant cancer cells to suppress the clearance of matrix-degrading proteases by LRP and thereby preserve an aggressive invasive phenotype [35]. In cultured podocytes in vitro, high glucose levels did not result in differences in the LRP expression level [82]. In addition, no differences were observed in circulating levels of LRP between control subjects and type 2 diabetic patients [83]. Thus the diabetes induced decrease of MMP activity appears not to be associated with differences in the expression level of LRP, but more on the increased availability of TIMPs or decreased activation due to increased levels of PAI-1. It still has to be determined if binding of CTGF to LRP indeed results in increased degradation and decreased availability of MMP-2 and MMP-9, or in decreased PAI-1 and TIMP degradation, resulting in a decreased gelatinase activity.

Binding of CTGF to integrins

Many of the effects of CTGF are thought to be mediated by its binding to integrins. Integrins are heterodimeric transmembrane receptors that were first identified based on their ability to bind and mediate cell adhesion to distinct components of the extracellular matrix [84, 85]. Although the integrin family was initially identified based on the role of family members in mediating cell adhesion, it has become clear that integrins also play important roles in initiating biochemical signals that contribute to a

wide variety of cellular responses, including survival, proliferation and migration [86]. In these responses, integrins function like other signaling receptors, initiating biochemical cascades of cytoplasmic protein modification and translocation as a consequence of binding to extracellular ligands (outside-in signaling) [86].

Integrins have been implicated in the regulation of the expression of MMPs, mainly in relation to the function of MMPs in tumor invasion [87]. However, the effect of integrins on MMPs is largely dependent on the specific integrin and the specific MMP. Treatment of glioblastoma and human breast cancer cells with antibodies against α3β1 and α5β1 integrin stimulated MMP-2 production which indicates that these integrins have a suppressing effect on MMP-2 [88, 89]. In contrast, α3β1 integrin stimulated MMP-9 activity in keratinocytes, which possibly involved stabilization of MMP-9 mRNA [90, 91], and αvβ6 integrin mediated secretion of MMP-9 in colon cancer cells [92]. In addition, in αvβ3 integrin enhanced MMP-2 secretion and activity in prostate cancer and melanoma cells [93, 94].

The expression level of integrins is influenced by the glucose concentration. In vitro, high glucose concentrations increased the expression of α5- and αvβ3 integrins on human glomerular epithelial cells, which was accompanied by decreased expression of α2, α3 and β1 integrins [95]. In vivo the expression of α3β1 integrin was decreased on podocytes of human patients with diabetes and in STZ-diabetic rats [96]. Also the expression of the αv integrin subunit in podocytes of diabetic rats was decreased [97]. Given the role of integrins in MMP regulation it seems plausible that decreased integrin expression due to diabetes is involved in deregulation of MMP(-activity) and consequent GBM-thickening.

CTGF mediates at least in part TGF-β-induced fibronectin synthesis by upregulation of the expression of α5β1 integrin in human mesangial cells [98], which also suppresses

Figure 1: Schematic representation of how CTGF might affect MMP-2 and MMP-9 activity. A "+" indicates stimulation and a "–" inhibition of a specific pathway by CTGF.

Interaction of CTGF with **TrkA** augments the TGF-β-induced Smad signaling by decreasing the expression of its inhibitor Smad7. Binding of CTGF to **LRP** might influence the degradation of MMPs as well as their inhibitors, the TIMPs, and PAI-1.

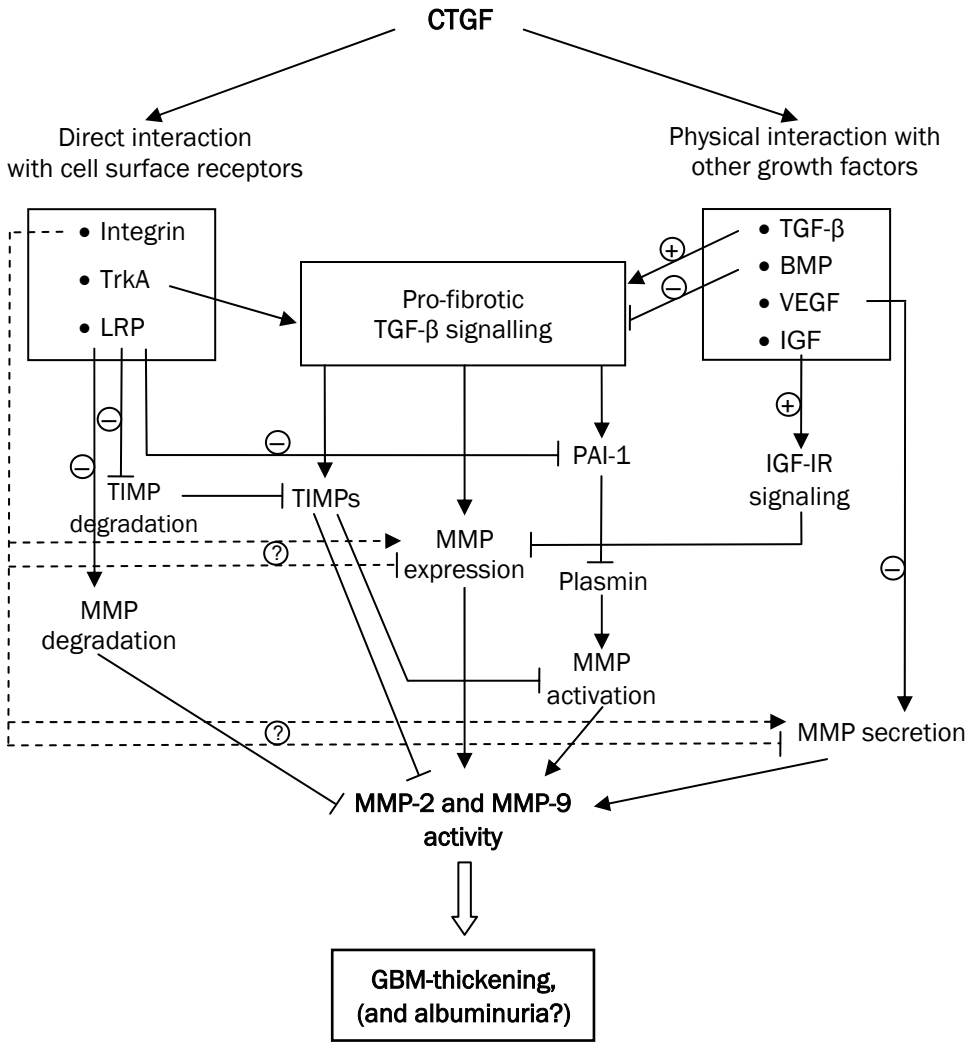
By binding to **TGF-β**, CTGF enhances TGF-β signaling while binding of CTGF to **BMP** reduces the BMP signaling. Together, this results in increased expression of MMPs as well as their inhibitors the TIMPs and PAI-1.

VEGF influences the secretion of MMPs. By binding of CTGF to **VEGF**, CTGF blocks the angiogenic action of VEGF. Presumably, this includes also the VEGF-dependent MMP secretion.

In cases of high ligand load, signaling of IGF-I via the IGF-IR inhibits the expression of MMPs. Binding of CTGF to **IGF** increases signaling via the IGF-IR, and might contribute to decreased MMP expression.

Finally, reduced plasmin level due to excess PAI-1 might frustrate activation of latent (pro-)MMPs. Binding of CTGF to **integrins** might influence the integrin dependent MMP expression and secretion, but this has not been addressed experimentally.

MMP-2 activity [88]. Interaction of CTGF with α5β1 integrin mediates enhanced cell adhesion of chondrocytes in vitro [99]. Furthermore, by binding to α5β1, CTGF stimulates fibrogenesis, adhesion, migration, and proliferation of pancreatic stellate cells [100]. Endothelial cell adhesion and migration is mediated by CTGF through integrin α5β3 [101] and binding of CTGF to this integrin (and HSPGs) also induces adhesion of rat activated hepatic stellate cells [57]. Finally interaction of CTGF with α6β1 integrin stimulates collagen deposition by gingival fibroblasts [102].



In summary, binding of CTGF to specific integrins influences integrin-signaling and integrin-mediated modulation of MMP activity. Diabetes-induced changes in integrin expression together with the modulating effect of CTGF-binding to specific integrins might thus be involved in the decreased matrix degrading activity of MMPs in renal tissue of diabetic mice.

Binding of CTGF to TrkA

Recently, CTGF has been shown to interact with the TrkA/neurotrophin receptor and with its general neurotrophin co-receptor $p75^{NTR}$ in mesangial cell cultures [103]. TrkA and $p75^{NTR}$ are known to be activated by the neurotrophin factor nerve growth factor (NGF) [104]. Neurothrophins are survival and differentiation factors in the nervous system, and although both receptors mediate the biologic effects of neurothrophins, it seems that TrkA plays the central role in this signaling while p75 participates in the formation of high affinity binding sites and enhanced NGF signaling via TrkA [104].

On binding and activation of TrkA by neurotrophins, the ligand-receptor complex is endocytosed into vesicles via a clathrin-dependent mechanism [105, 106]. Also CTGF can be endocytosed from the cell surface into endosomes, from which the growth factor translocates into the nucleus [107]. Given the interaction of CTGF with TrkA and $p75^{NTR}$, endocytosis of CTGF might thus involve binding to these factors receptors.

In addition to expression in human mesangial cells in vitro [103], TrkA is highly expressed in the adult rat kidney [108]. In humans, renal TrkA expression was observed in tubuli but it was not detectable in glomeruli [109]. Diabetic patients showed increased TrkA expression in the skin compared to its control subjects [110]. So far no data are present on TrkA expression level, localization, and signaling activity in diabetic kidneys in vivo.

It has been speculated that CTGF may acts to fine-tune the TGF-β/Smad signaling pathway by regulation of its inhibitor Smad7 [103]. Smad7 expression can be repressed by TIEG-1 (TGF-β immediate early gene-1) by binding to a GC-box in the promoter region of the Smad7 gene [111]. Wahab et al. reported that CTGF is able to rapidly induce the expression of TIEG-1 in human mesangial cells *in vitro* [112]. CTGF-dependent upregulation of TIEG-1 requires tyrosine kinase activity of the neurothrophin receptor TrkA [103]. In combination with the rapid turnover of Smad7 by the ubiquitin-proteosome pathway [113], the repression of Smad7 transcription due to immediate early expression of TIEG-1 might explain the observed close temporal correlation between the increase of TIEG-1 and the decrease of Smad7 upon stimulation with CTGF. As described above, increased signaling via the TGF-β/Smad pathway results in increased expression of TIMPs and PAI-1 as well as CTGF itself, which might contribute to decreased activity of MMP-2 and MMP-9.

Thus, repression of Smad7 by TIEG-1 due to interaction of CTGF with TrkA might lead to enhanced TGF-β signaling via the Smad pathway and thus contribute to the CTGF-dependent decrease of MMP activity and consequent GBM thickening in the diabetic kidney.

In conclusion, the CTGF dependent GBM thickening and reduction of MMP activity in the diabetic kidney might involve several different mechanisms, which may account also for the CTGF-dependent basement membrane thickening in the diabetic retinal and other vasculature. These include physical interaction of CTGF with other growth factors, thus modulating their action, and direct interaction of CTGF with cell surface receptors, as well as combinations thereof. From the *in vivo* impact of reduced CTGF expression on renal MMP activity in diabetic mice, it has become apparent that the ultimately negative effect of CTGF on regulation of MMP activity, may derive largely from its impact on post-translational events like secretion and activation of (pro-)MMPs. In contrast, at the level of MMP (mRNA) expression, the balance between stimulation by e.g. TGF-β, and inhibition by IGF-R signaling, although both subject to enhancement by CTGF, appears not to be affected significantly by CTGF overexpression.

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Chapter 9

General discussion and summary

Diabetic nephropathy (DN) is a common complication of diabetes mellitus (DM). About 25% to 40% of the patients with DM will eventually develop diabetic nephropathy, mostly between 15-20 years after diagnosis [1-3]. In a large part of the world DN is the major cause of end stage renal disease, necessitating dialysis or a kidney transplantation.

DN is a progressive fibrotic kidney disease that is structurally characterized by mesangial matrix accumulation and thickening of the glomerular basement membrane (GBM). In later stages also tubulointerstitial fibrosis is prominent. Functional characteristics of DN include albuminuria and progressive decrease of the glomerular filtration rate (GFR). Hyperglycemia and hypertension are known risk factors for development and progression of DN. Both these factors are able to induce the important pro-fibrotic growth factor transforming growth factor β (TGF-β). Experiments in animal models of DN have shown that inhibition of TGF-β can slow down or prevent development of DN. However, as TGF-β is also involved in important anti-inflammatory and anti-proliferative processes, inhibition of TGF-β is not an attractive therapeutic strategy for DN.

Connective tissue growth factor (CTGF) is an important downstream mediator in the fibrotic action of TGF-β. CTGF is upregulated by TGF-β as well as by the diabetic milieu, which is characterized by hyperglycemia and advanced glycation end products (AGEs), reactive oxygen species (ROS), and by mechanical strain (hypertension). During embryogenisis, CTGF is critically involved in skeletal development and CTGF-null mice are not viable because of ribcage defects that make breathing impossible [4]. In adult animals, however, so far no negative effects of reduction of CTGF levels have been observed.

The aim of this thesis has been to study the role of CTGF in the development of diabetic nephropathy, with a special focus on the potential of CTGF as marker, pathogenic factor and target for therapeutic intervention for DN.

The pathogenesis and main characteristics of DN and the scope of this thesis are introduced in **chapter 1**, and a broad overview of the current knowledge about CTGF and its role in DN is presented in **chapter 2**.

CTGF AS A MARKER FOR DN

Increased expression of CTGF mRNA and protein in renal tissue of patients with DN was already reported at the start of our investigations. Therefore, it was suggested that CTGF might be usable as a marker for DN. However, a clinically useful marker for DN needs to be detectable in easily available patient samples like plasma and urine. Ideally, the marker should identify individuals that will develop DN before the disease has become manifest. Thus, marker levels in patients at risk for DN need to be clearly different from those in patients without an increased risk for DN.

To investigate the potential of CTGF as clinical marker for DN we measured CTGF levels in plasma of diabetic patients with and without nephropathy and studied the relation of these levels with general patient characteristics and markers for nephropathy. Therefore we determined the plasma levels of full length CTGF as well as its N-terminal fragment (CTGF-N) in control subjects and patients with diabetes.

In **chapter 3** we present a small pilot study of plasma CTGF-N levels of type 1 diabetic patients with and without nephropathy. Levels of full length CTGF were below the detection limit in the majority of the subjects tested, while CTGF-N was readily detectable in most plasma (and urine) samples. The very low levels of circulating full length CTGF might be caused by the known matrix binding capacity of the C-terminal part of CTGF [5, 6]. Since CTGF can be cleaved by several proteases [7, 8], CTGF-N might be released from the matrix and enter the circulation, which might explain its elevated presence in plasma and urine samples. Plasma CTGF-N levels were observed to be significantly higher in patients with diabetic nephropathy than in diabetic patients without nephropathy and healthy control subjects. Another observation in this study was that plasma CTGF-N levels in diabetic patients correlated with albuminuria and creatinine clearance, which are important determinants of DN. Due to the small group size, no conclusions could be drawn about the relation of CTGF-N with other parameters in patients with DN.

We proceeded by analyzing a large cohort of diabetic patients with and without nephropathy (**chapter 4**). Also in that study, CTGF-N levels were significantly elevated in diabetic patients with nephropathy compared to normoalbuminuric diabetic patients. Moreover, plasma CTGF-N level correlated with severity of albuminuria and loss of creatinine clearance within the group of patients with DN. The odds ratio for DN of a 1 SD increase of plasma CTGF-N level was found to be similar to that of HbA_{1c} and hypertension.

From these patient studies we conclude that plasma CTGF-N levels are increased in diabetic patients with DN and that these levels are associated with severity of DN. In other studies, it was shown that also urinary CTGF-N excretion is associated with severity of DN [9-11]. These data confirm that CTGF might be a useful marker for development and progression of DN. However, longitudinal studies will be needed to elucidate the possible clinical value of determination of CTGF levels in plasma and urine as a prognostic factor for development and progression of DN.

CTGF AS PATHOGENIC FACTOR AND TARGET FOR THERAPEUTIC INTERVENTION IN DN

To elucidate the role of CTGF as pathogenic factor in DN, we studied the temporal expression profile and distribution of CTGF in experimental diabetic mice in **chapter 5**. The most important observation in that study was that CTGF is significantly increased in plasma and urine of diabetic mice and that urinary CTGF excretion correlated with albuminuria, the main characteristic of DN. In addition, CTGF mRNA expression was increased in kidney, heart and liver tissue of diabetic mice. Using immunohistochemical staining by a unique monoclonal antibody against an epitope of the N-terminal half of the CTGF molecule, we identified the podocyte as the main source of diabetes-induced renal CTGF overexpression. This is an important addition to previous observations that *in vitro* CTGF expression is increased in mesangial cells stimulated with TGF-β, high glucose or AGEs [12, 13] and that *in vivo* renal CTGF expression is increased in diabetic patients and experimental animals [12, 14, 15].

The fact that increased CTGF expression was observed in the kidney of diabetic patients [14] and animals (see also [12, 15]), indicates that the kidney might be the source of elevated levels of plasma- and urinary CTGF. Increase of plasma CTGF due to renal overexpression or dysfunction might even be expected to contribute to cardiovascular deterioration in the context of (diabetic) nephropathy. However, our investigations revealed that local CTGF expression is increased also in other organs (including the heart) of diabetic mice, and it seems likely that these too contribute to increased CTGF levels in plasma and urine. In addition, decreased GFR, and proteinuria or tubular dysfunction might also lead to increased CTGF levels in plasma and urine, respectively. Because no detailed analyses are available of plasma and urinary CTGF levels in the same group of diabetic patients with DN, the exact influence of proteinuria and decreased GFR is still unknown. Also our observation in diabetic mice, that CTGF mRNA expression is increased already shortly after induction of diabetes and is accompanied by increased urinary CTGF excretion, cannot answer this question, since we did not obtain samples before day 7 when both albuminuria and CTGF increase were evident already.

Notwithstanding these limitations, the results of this study do suggest, (or are at least compatible with) a pathogenic role of CTGF in the development of complications of DM, which is further substantiated in the next chapters (**chapters 6 and 7**).

The role of CTGF in the development of DN was further studied in **chapter 6**, which describes the impact of genetic disruption of one of both CTGF alleles as a model for anti-CTGF therapy. In these experiments we show that CTGF heterozygous mice ($CTGF^{+/-}$), have lower CTGF levels in plasma and urine as well as lower CTGF mRNA expression compared to CTGF wild type ($CTGF^{+/+}$) mice, under diabetic as well as under control conditions. The lower levels of CTGF in heterozygous $CTGF^{+/-}$ diabetic mice were accompanied by significantly lower albuminuria compared to diabetic wild type $CTGF^{+/+}$

mice, and by complete absence of GBM thickening. This led us to conclude that reduction of CTGF availability in diabetes appears to limit the severity of diabetic nephropathy.

To elucidate the cause of the striking absence of GBM thickening in $CTGF^{+/-}$ mice, we first compared the mRNA and protein expression of several GBM components reported to be altered in DN. Unexpectedly, this did not reveal significant differences between diabetic $CTGF^{+/-}$ mice as compared to diabetic $CTGF^{+/+}$ mice. Since the thickness of the GBM is determined not only by production of GBM components but also by the rate of ECM degradation by proteolytic enzymes, we compared the matrix degrading activity of matrix metalloproteinase (MMP)-2 and -9 (the "gelatinases") in the mice with and without GBM thickening. This gelatinase activity was decreased in diabetic $CTGF^{+/+}$ (wild type) mice (in agreement with [16]), but remained completely unaltered in diabetic $CTGF^{+/-}$ mice. It thus appears that increased expression of CTGF decreased the turnover of the GBM by reduction of the gelatinase activity, resulting in increased GBM thickness. Control experiments with 1,10-phenanthroline monohydrate showed that the gelatinase activity detected in our assays represents MMP activity. So far the mechanism of this CTGF-dependent decrease in MMP activity is unknown. For an overview of possible pathways involved in CTGF-dependent down regulation of MMP-activity in DM, the reader is referred to **chapter 8.**

CTGF INDUCED BASEMENT THICKENING IN DIABETIC RETINOPATHY

In diabetic patients thickening of vascular basement membranes is a general phenomenon, not restricted to diabetic nephropathy. Also in diabetic retinopathy, an even more common complication of diabetes, basement membrane thickening is one of the main characteristics.

Therefore, we also measured basement membrane thickness of retinal vessels in $CTGF^{+/-}$ and $CTGF^{+/+}$ mice under control and diabetic conditions (**chapter 7**). In agreement with our observations in the kidney, also the thickening of the basement membrane of retinal capillaries was prevented in diabetic $CTGF^{+/-}$ mice. Unfortunately, retinal tissue availability was too limited to allow for measurement of gelatinase activity. As for the source of CTGF in the retina, local CTGF mRNA expression is known to be increased in the eye in diabetes [17, 18], but also the high levels of plasma CTGF derived from overproduction or malfunction of other organs, including the kidney, might have contributed to CTGF-dependent basement membrane thickening of retinal vessels. Since basement thickening is involved in important diabetes-induced retinal vascular deterioration such as apoptosis of pericytes and endothelial cells, our observations are likely to have important implications also for understanding of more advanced stages of diabetic retinopathy [19, 20].

CONCLUSION AND PERSPECTIVE

Together, the data presented in this thesis indicate that CTGF might be a useful marker for DN, a pathogenic factor in diabetic tissue remodeling, and a potential therapeutic target for prevention and treatment of DN. In addition, CTGF was found to be upregulated in other organs in diabetes and the possible beneficial effects of CTGF-inhibition on diabetic nephropathy might be extrapolated to e.g. diabetic retinopathy and other fibrotic disease. In a recent clinical trial it was found that a CTGF-neutralizing antibody (FG-3019) was safe and well tolerated [21]. Currently, diabetic patients with microalbuminuria are being recruited for a clinical trial to assess whether treatment with CTGF-neutralizing antibodies can help to prevent progression to diabetic nephropathy or even revert microalbuminuria to normoalbuminuria.

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Chapter 10

Samenvatting in het Nederlands

DIABETES MELLITUS

Diabetes mellitus, in de volksmond beter bekend als suikerziekte of diabetes, is een chronische stofwisselingsziekte waarbij het lichaam de bloedsuikerspiegel niet meer binnen de normale grenzen kan houden. Dit wordt veroorzaakt door een probleem met insuline, een eiwit dat een belangrijke rol speelt in de regulatie van de bloedsuikerspiegel. Insuline wordt geproduceerd in gespecialiseerde cellen van de Eilandjes van Langerhans in de alvleesklier. Een groot deel van de andere cellen in het lichaam heeft insuline nodig voor de opname van glucose uit het bloed. Het opgenomen glucose wordt vervolgens gebruikt als brandstof voor de energiebehoefte van de cel of het wordt in de cel opgeslagen voor later gebruik. Als er te weinig insuline wordt aangemaakt in de alvleesklier of als de insuline zijn werk niet goed kan doen stijgt het glucosegehalte in het bloed en ontstaat diabetes mellitus.
Er zijn 2 verschillende typen diabetes mellitus. Bij patiënten met type 1 diabetes zijn de insuline producerende cellen beschadigd, meestal door een verkeerde reactie van ons eigen immuunsysteem. Door de vernietiging van de insulineproducerende cellen produceert het lichaam geen of te weinig insuline en bij deze patiënten is het toedienen van insuline dan ook noodzakelijk. Gebeurt dat niet dan kan de patiënt in coma raken en overlijden. Bij type 2 diabetes maakt de patiënt nog wel insuline maar is de gevoeligheid van de cellen voor insuline verminderd. Een van de belangrijkste risicofactoren voor type 2 diabetes is ernstig overgewicht. Daarnaast kunnen leeftijd en genetische factoren een rol spelen. Omdat de patiënt zelf nog wel insuline maakt zijn de symptomen milder dan bij type 1 diabetes en wordt de ziekte meestal niet direct opgemerkt.
Bij beide typen diabetes ontstaan te hoge bloedsuikerspiegel en wordt de urine van de patiënt “honingzoet” (In het Latijn: mellitus). Door de hoge glucosespiegel in de urine (glucosurie) is de nier niet in staat om de urine goed te concentreren. Hierdoor neemt het volume dat de patiënt uitplast toe (polyurie). Door deze overmatige urineproduktie verliest het lichaam veel water en krijgt de patiënt dorst. Naast problemen met de opname van glucose uit de bloedbaan treden er bij diabetes ook grote veranderingen in het metabolisme van eiwitten en vetten op.

Naast acute veranderingen in het lichaam kan diabetes ook op de lange termijn tot ernstige gezondheidsproblemen leiden. Door de chronische verhoging van de bloedsuikerspiegel raken de bloedvaten beschadigd. Hierdoor lopen diabetespatiënten een verhoogde kans op het ontwikkelen van hart- en vaatziekten. Deze hart- en vaatziekten zijn bij 50 – 80% van de patiënten met diabetes de uiteindelijke oorzaak van overlijden. Andere veel voorkomende complicaties van de diabetes zijn oogafwijkingen (retinopathie), zenuwafwijkingen (neuropathie) en nierproblemen (nefropathie). Ook bij deze complicaties speelt schade aan (kleine) bloedvaten een

belangrijke rol. Bovendien hebben met name diabetespatiënten met nierproblemen een vele malen hogere kans om aan hart- en vaatziekten te overlijden.

DIABETISCHE NEFROPATHIE

Diabetische nefropathie is een veel voorkomende complicatie van diabetes mellitus. Ongeveer 25% tot 40% van alle diabetespatiënten ontwikkeld op den duur diabetische nefropathie, meestal tussen 15 en 20 jaar na de diagnose van diabetes. De schade aan de nier kan uiteindelijk zo erg zijn dat eind-stadium nierfalen ontstaat en de patiënt aangewezen is op dialyse of op een niertransplantatie.
Diabetische nefropathie is een progressieve aandoening van de nier die gekenmerkt wordt door de aanmaak van littekenweefsel in de glomerulus (de vaatkluwen in de nier waar het bloed gefilterd wordt en urine geproduceerd wordt) en verdikking van de glomerulaire basaalmembraan (de laag die het bloed van de urine scheidt en waarover het bloed gefiltreerd wordt). Daarnaast neemt de hoeveelheid eiwit en met name albumine in de urine toe (albuminuria). Uiteindelijk neemt de filtratiecapaciteit van de nier af en ontwikkelt de patiënt eindstadium nierfalen. De belangrijkste risicofactoren voor het ontstaan van diabetische nefropathie zijn te hoge bloedsuikerspiegels en hoge bloeddruk.
Transforming growth factor-β (TGF-β) is een eiwit dat bij diabetes verhoogd wordt aangemaakt. Er is aangetoond dat TGF-β een belangrijke rol speelt bij het ontstaan van nieraandoeningen. Het zorgt voor nierfibrose; schadelijke littekenvorming in de nieren. In proefdierstudies is aangetoond dat vermindering van de hoeveelheid TGF-β het ontstaan van diabetische nefropathie kan vertragen of zelfs voorkomen. Maar TGF-β is ook nog bij andere, gunstige processen in het lichaam betrokken zoals het bestrijden van tumoren en infecties. Muizen waarin TGF-β uitgeschakeld is ontwikkelden weliswaar geen diabetische nefropathie, maar de levensduur van deze dieren was zeer beperkt door het ontstaan van ernstige ontstekingsreacties en tumoren. Het uitschakelen van TGF-β lijkt dus geen geschikte strategie te zijn om diabetische nefropathie te voorkomen.

CONNECTIVE TISSUE GROWTH FACTOR (CTGF)

Recent is nog een ander eiwit ontdekt dat een rol speelt in diabetische nieraandoeningen: connective tissue growth factor (CTGF). Net als TGF-β is CTGF een zogenaamde “groeifactor”, een eiwit dat na produktie door de cel wordt uitgescheiden en vervolgens een effect heeft op andere cellen. CTGF is oorspronkelijk ontdekt in atherosclerotische beschadigingen van de bloedvaten. Maar ook in allerlei andere organen is de aanwezigheid van CTGF aangetoond. TGF-β is een belangrijke stimulus voor de produktie van CTGF, maar ook van diabetische condities (hoge bloedsuikerspiegels en versuikerde eiwitten (advanded glycation end products; AGEs))

en een hoge bloeddruk is bekend dat ze kunnen leiden tot een verhoogde produktie van CTGF.
Na de produktie wordt het CTGF door de cel uitgescheiden in de extracellulaire matrix, het geheel van eiwitten dat de cellen omringt en bij elkaar houdt. Van CTGF is aangetoond dat het kan binden aan bepaalde componenten van de extracellulaire matrix. Daarnaast is bekend dat het ook een interactie aan kan gaan met verschillende andere groeifactoren, zoals TGF-β, BMP, IGF-I en VEGF. Van al deze groeifactoren is bekend dat ze een effect hebben op de aanmaak van bindweefseleitwitten door de cel. Door binding van deze groeifactoren kan CTGF de werking van deze groeifactoren beïnvloeden. Over het algemeen leidt dit tot een toename van de hoeveelheid bindweefseleiwitten die bijdragen aan de toename van littekenweefsel tussen de cellen (fibrose). Daarnaast is bekend dat CTGF kan binden aan bepaalde eiwitten op de cel. Het is nog niet precies bekend hoe binding van CTGF aan deze cel-eiwitten het functioneren van de cel beïnvloed maar waarschijnlijk leidt een deel van deze interacties ook tot een verhoogde aanmaak van bindweefseleiwitten. In de nier kan de fibrose de filtratie van bloed in de glomerulus verstoren en uiteindelijk leiden tot afname van de nierfunctie.
In tegenstelling tot TGF-β zijn er van CTGF geen anti-tumor en ontstekingsremmende functies aangetoond en zou het wellicht dus zonder gevaar kunnen worden uitgeschakeld. Daarmee lijkt CTGF een interessant aangrijpingspunt voor de ontwikkeling van nieuwe medicijnen om nefropathie te voorkomen, of progressie tegen te gaan.

In dit onderzoek hebben wij de rol van CTGF in het ontstaan van diabetische nieraandoeningen nader bekeken. Hierbij hebben wij met name gekeken naar de potentie van CTGF als marker, als pathogene factor en als aangrijpingspunt voor therapie van diabetische nefropathie. Allereerst wordt in hoofdstuk 2 een uitgebreid overzicht gegeven van de huidige kennis op dit gebied.

CTGF ALS MARKER VOOR DIABETISCHE NEFROPATHIE

Op dit moment wordt albuminurie gebruikt als belangrijkste marker voor diabetische nefropathie. Als de hoeveelheid albumine in de urine boven de 30 mg/24 uur uitkomt (maar onder de 300 mg/24 uur blijft) heeft een patiënt microalbuminurie en wordt er aangenomen dat de kans groot is dat deze patiënt nefropathie ontwikkeld. Echter, patiëntenstudies toonden aan dat ongeveer $^2/_3$ van de patiënten met microalbuminurie geen nefropathie ontwikkeld. Zij blijven lange tijd microalbuminurisch of hun albumine uitscheiding daalt zelfs weer naar het niveau in gezonde patiënten (onder de 30 mg/24 uur). Door alleen naar de microalbuminurie te kijken zijn de patiënten die een verhoogd risico lopen op de ontwikkeling van diabetische nefropathie dus niet goed te

identificeren. Dit leidt er toe dat een deel van hen niet optimaal (preventief) of juist onnodig behandeld wordt.
Van CTGF was al bekend dat het verhoogd aanwezig is in weefsel van patiënten met diabetes en in het bijzonder in de nier van patiënten met diabetische nefropathie. Om uit te zoeken of CTGF geschikt is als marker voor diabetische nefropathie hebben wij in hoofdstuk 3 en 4 de hoeveelheid CTGF in het bloed van patiënten met diabetes met en zonder nefropathie gemeten. Hieruit bleek dat de hoeveelheid CTGF in het bloed van patiënten met diabetische nefropathie hoger was dan in het bloed van diabetes patiënten zonder nefropathie en van gezonde controlepersonen. Daarnaast bleek dat de hoeveelheid CTGF in het bloed van deze patiënten verband houdt met de ernst van de nierziekte. In het bloed van patiënten met microalbuminurie daarentegen, was er een hele grote spreiding in de hoeveelheid CTGF. Het is heel goed mogelijk dat de microalbuminurische patiënten met veel CTGF in hun bloed degenen zijn die een grote kans hebben om later nefropathie te ontwikkelen. Studies waarin patiënten over een langere tijd gevolgd worden zullen aan moeten tonen of verhoogde CTGF spiegels inderdaad gebruikt kunnen worden om te voorspellen welke patiënten een verhoogd risico lopen op de ontwikkeling van nefropathie.

CTGF ALS PATHOGENE FACTOR EN TARGET VOOR THERAPIE

Uit eerder onderzoek en uit de literatuur was al bekend dat CTGF betrokken is bij fibrose. Van hoge bloedsuikerspiegels, hoge bloeddruk en ook van TGF-β is bekend dat het de aanmaak van CTGF kan induceren. Hierdoor neemt de hoeveelheid “bindweefsel” tussen de cellen toe waardoor de functie van organen afneemt. Organen die erg vatbaar zijn voor deze bindweefselvorming zijn de nier en het netvlies in het oog, maar ook hart en bloedvaten worden op deze manier aangetast.
In hoofdstuk 5 hebben wij studies met zowel type 1 als type 2 diabetische muizen uitgevoerd waarin werd aangetoond dat de hoeveelheid CTGF in bloed en urine al heel snel na de inductie van diabetes verhoogd is ten opzichte van gezonde muizen. Tevens bleek dat de hoeveelheid CTGF die uitgescheiden wordt in de urine verband houdt met de ernst van de nierproblemen. Daarnaast toonden wij aan dat CTGF in hoge mate in de zieke nier zelf geproduceerd wordt en dat dit met name gebeurt in de podocyt, een cel die erg belangrijk is in het proces van filtratie van het bloed tot urine.

Om te onderzoeken of verhoogde CTGF spiegels noodzakelijk zijn voor de veranderingen in de nier bij diabetische nefropathie hebben wij vervolgens in hoofdstuk 6 een studie uitgevoerd in CTGF$^{+/-}$ muizen. Dit zijn muizen waarin op een van beide chromosomen het CTGF gen uitgeschakeld is. Hierdoor hebben deze muizen maar **éé**n functioneel CTGF gen in plaats van twee en kunnen zij onder bepaalde omstandigheden minder CTGF maken. In CTGF$^{+/-}$ muizen was de CTGF spiegel in bloed

en urine en ook de aanmaak van CTGF in de nier, nadat zich diabetes ontwikkeld had, niet of nauwelijks verhoogd ten opzichte van die in gezonde muizen. Deze lagere CTGF spiegels in diabetische $CTGF^{+/-}$ muizen werden vergezeld van een significante vermindering van de uitscheiding van albumine door de nier, een belangrijke maat voor de ernst van de diabetische nierziekte. Daarnaast was er ook een significant verschil in de dikte van de glomerulaire basaalmembraan (GBM), de eiwitlaag die fungeert als filter tussen bloed en urine, en die in nauw contact staat met de CTGF-producerende podocyt. Van veranderingen in de GBM is bekend dat ze (deels) verantwoordelijk zijn voor verhoogde albumine uitscheiding door de nier. De verdikking van de GBM die "normaal" optreedt bij diabetes bleek niet voor te komen in diabetische $CTGF^{+/-}$ muizen. Hieruit kan geconcludeerd worden dat CTGF direct betrokken is bij de verdikking van de GBM en dus beschouwd kan worden als pathogene factor voor diabetische nefropathie. Naast verschillen in verdikking van de GBM bleek in hoofdstuk 7 de basaalmembraan van de bloedvaatjes in het netvlies van diabete $CTGF^{+/-}$ muizen ook minder verdikt te zijn dan in diabete $CTGF^{+/+}$ muizen. Dit suggereert dat hoge CTGF spiegels niet alleen in de nier maar ook op andere plaatsen in het lichaam een rol spelen bij basaalmembraan verdikking in diabetische complicaties.

De dikte van de GBM is afhankelijk van zowel de produktie als de afbraak van de eiwitten waaruit deze laag opgebouwd is. Meting van de produktie van een aantal eiwitten waarvan bekend is dat ze onderdeel uitmaken van de GBM liet, ondanks dat de produktie van een deel van deze eiwitten verhoogd was in diabetische muizen, geen duidelijke verschillen zien tussen diabetische $CTGF^{+/+}$ en diabetische $CTGF^{+/-}$ muizen (hoofdstuk 6). Het lijkt er dus op dat het verschil in de dikte van de GBM in deze diabete muizen niet of nauwelijks bepaald wordt door een verhoogde aanmaak van deze eiwitten. Daarom hebben we vervolgens onderzocht of een verschil in "turnover" van de GBM een verklaring voor het verschil in dikte zou kunnen zijn.
Eiwitten die een belangrijke rol spelen in de afbraak van bepaalde eiwitten in de GBM zijn de matrix metalloproteïnases (MMPs). Met name van MMP-2 en MMP-9 is beschreven dat ze een belangrijke rol spelen in de afbraak van de eiwitten in de GBM. In nieren van gezonde en diabetische muizen bleek dat deze MMPs vooral actief zijn in de glomerulus, daar waar het bloed gefilterd wordt en waar ook de hoeveelheid CTGF verhoogd is bij diabetes. Verder bleek dat in diabetische $CTGF^{+/+}$ muizen de activiteit van deze MMPs lager was dan die in gezonde en in diabetische $CTGF^{+/-}$ muizen. Het lijkt er dus op dat bij diabetes door middel van verhoging van de hoeveelheid CTGF, de MMP activiteit onderdrukt wordt, wat uiteindelijk resulteert in verdikking van de GBM en mogelijk ook in de verergering van de albuminurie.
Het mechanisme achter de verlaging van de MMP activiteit door CTGF is tot nu toe onbekend. In hoofdstuk 8 van dit proefschrift wordt een overzicht gegeven van de

manieren waarop dit theoretisch gezien zou kunnen werken. Hierbij wordt een overzicht gegeven van de interacties die CTGF met eiwitten en cellen in de nier aan kan gaan en hoe dit mogelijkerwijs de hoeveelheid en de activiteit van MMP-2 en MMP-9 zou kunnen veranderen. Echter, verdere studies zijn nodig om aan te tonen of dit ook werkelijk op deze manier plaatsvindt.

Samenvattend blijkt uit dit onderzoek dat CTGF en diabetische nierproblemen nauw met elkaar in verband staan en dat remming van CTGF inderdaad leidt tot minder nierschade. Naast de mogelijkheden om CTGF als marker te gebruiken blijkt het inderdaad direct betrokken te zijn bij het ontstaan van diabetische nefropathie en heeft het aanpakken van CTGF tevens potentie als therapie voor het voorkomen en behandelen van diabetische nefropathie.
Verdere studies in muizen waarin CTGF volledig uitgeschakeld kan worden en klinische trials met CTGF-neutraliserende antistoffen in patiënten, moeten aantonen of uitschakelen van CTGF inderdaad het ontstaan van diabetische nefropathie kan voorkomen en of anti-CTGF therapie in patiënten met diabetes niercomplicaties zou kunnen voorkomen of zelfs zou kunnen helpen genezen.

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En toen waren er nog 4 lege bladzijden die mij aanstaarden. Vier bladzijden die nog “even” gevuld moesten worden. En hoewel ik ze tot het allerlaatste moment bewaard heb, niet precies wetende hoe ik ze moest vullen (maar wel zeker wetend dat ze echt wel vol zouden komen) zijn het waarschijnlijk wel de bladzijden die het vaakst uitgespeld gaan worden. Nou hup, computer op schoot (sorry Bolle, jij moet even opzij), portje erbij, tijd om sentimenteel te gaan doen.

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Peggy

Curriculum vitae

De auteur van dit proefschrift werd geboren op 27 augustus 1973 te Geleen. In 1991 behaalde zij haar VWO diploma aan het Dr. Mollercollege te Waalwijk. In datzelfde jaar begon ze aan de studie Medische biologie aan de Universiteit van Utrecht. Tijdens haar opleiding deed zij haar hoofdvakstage bij de afdeling Virologie van de faculteit der Diergeneeskunde onder begeleiding van Drs. C.A.M. de Haan en Prof. Dr. P.J. Rottier. Haar bijvakstage deed zij bij de afdeling Interne geneeskunde van het Academisch Ziekenhuis Utrecht onder begeleiding van Dr. F. de Coo en Prof. Dr. B.S. van Asbeck. Daarnaast had zij tijdens haar studie enkele nevenfunctie als studentassistent. In februari 1997 behaalde zij haar doctoraal examen. Vervolgens werkte zij gedurende 2 jaar als administratief medewerkster en medewerker prognose & analyse bij de afdeling Voedingsplanning van het UMC Utrecht. In 2000 keerde zij terug in het wetenschappelijk onderzoek. In dat jaar werkte zij bij de afdeling Veterinaire Farmacologie, Farmacie en Toxicologie aan het onderzoek naar de mutageniciteit van Ochratoxine A van Drs. A.Y. Simarro-Doorten en Prof. Dr. J. Fink-Gremmels. In 2001 was zij vervolgens enige tijd werkzaam bij de afdeling Medische Genetica van het UMC Utrecht. Onder supervisie van Dr. C. Wijmenga deed zij daar onderzoek naar de genetische achtergrond van Indian Childhood Cirrhosis. In de periode december 2001 - 2006 werkte zij bij de afdeling Pathologie van het Universitair Medisch Centrum te Utrecht onder begeleiding van Dr. R. Goldschmeding en Dr. F.A. van Nieuwenhoven aan haar promotieonderzoek. De resultaten van dit onderzoek staan beschreven in dit proefschrift. Daarnaast volgde zij in deze periode ook de SMBWO-opleiding tot Experimenteel pathobioloog.