Connective Tissue Growth Factor and Bone Morphogenetic Proteins in Diabetic Nephropathy

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Connective Tissue Growth Factor and Bone Morphogenetic Proteins in Diabetic Nephropathy

Connective Tissue Growth Factor en Bone Morphogenetic Proteins in Diabetische Nefropathie

(met een samenvatting in het Nederlands)

Proefschrift

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Tri Quang Nguyen

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Promotoren: Prof.dr. P.J. van Diest
Prof.dr. G.J.A. Offerhaus

Co-promotor: Dr. R. Goldschmeding

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Ἓν οἶδα ὅτι οὐδὲν οἶδα
Σωκράτης

I know one thing, that I know nothing
Socrates

Aan mijn familie

Contents

Chapter I

General Introduction

Diabetic Nephropathy

Epidemiology

Diabetes mellitus is characterized by inappropriately high blood glucose levels resulting from low levels of insulin (type 1 diabetes) and/or from abnormal resistance to the effects of insulin (type 2 diabetes). The diabetes epidemic is a rapidly growing problem in health care, accounting for approximately 150 million patients worldwide. It is expected that in 2025, the number of diabetic patients will have reached 300 million. In the Netherlands, this will result in a total of more than 350 thousand diabetic patients, which is equivalent to a prevalence of 2.7% in adults older than 20 years (1). Patients with diabetes have an increased risk to develop macrovascular and microvascular complications, including atherosclerosis, retinopathy, neuropathy, and nephropathy. The onset of diabetic nephropathy is typically between 10 and 15 years after the onset of diabetes (2;3). Currently, diabetic nephropathy is the most important cause of end-stage renal disease (ESRD) and contributes significantly to mortality in renal patients (4). Moreover, diabetic patients with ESRD undergoing dialysis have a mean life expectancy of only months to years (5).

Prior to the current period of intensive monitoring and treatment of patients with diabetes, it was estimated that approximately 25–40% of diabetic patients eventually developed diabetic nephropathy (6;7). As a result of aggressive antihypertensive medication and strict glycemic control, cumulative incidence of diabetic nephropathy has decreased over the past decades (8;9). For instance, among the patients who developed diabetes from 1966 to 1970, the cumulative incidence of diabetic nephropathy after 25 years of diabetes was 8.9%, as compared to 30.0% among the patients who developed diabetes in the period from 1961 to 1965 (10). Although the natural course is more variable in patients with type 2 diabetes, recent data suggest that the risk to develop diabetic nephropathy and progression to ESRD is currently comparable in the two types of diabetes (11).

Manifestation

The earliest clinical sign of renal injury in patients with diabetes is the development of microalbuminuria, which is defined as urinary albumin excretion (UAE) between 30 and 300 mg per day. After a mean duration of diabetes of 14 years, microalbuminuria is present in approximately 28% of diabetic patients (12). Microalbuminuria may remain stable or even regress in a substantial proportion of these patients. However, depending on the time at which microalbuminuria develops, up to 50% of diabetic patients with microalbuminuria progresses to overt proteinuria/macroalbuminuria, which is defined as UAE higher than 300 mg per day (13;14). Clinically, diabetic nephropathy is diagnosed by the presence of macroalbuminuria and it is often accompanied by decline in glomerular filtration rate (GFR).

The pathological changes in diabetic nephropathy are characterized by excessive accumulation of extracellular matrix (ECM) components in both glomeruli and the tubulointerstitial area. On the histological level, diabetic nephropathy is indicated by the presence of mesangial expansion, diffuse and nodular glomerulosclerosis, hyaline arteriolosclerosis, and tubulointerstitial fibrosis (Figure 1). On the ultrastructural level, the changes of diabetic nephropathy consist of thickening of the glomerular basement

membrane (GBM), reduction in the number of podocytes, and broadening of podocyte foot processes with reduction in the density of open slit pores between podocytes (15;16). Diabetic kidneys also show reduced expression of nephrin and decreased activity of matrix metalloproteinases (17;18).

Pathogenesis

The pathophysiology of diabetic nephropathy is complex and involves an interaction between metabolic and hemodynamic factors (19). The metabolic factors are mainly related to elevated blood glucose levels. Hyperglycemia induces a defect in the mitochondrial electron transport chain, which results in increased production of reactive oxygen species and oxidative stress. The current concept is that the effects of hyperglycemia and the changes observed in diabetic nephropathy are essentially mediated by increased oxidative stress (20).

Hyperglycemia might promote the development of diabetic nephropathy by direct induction of mesangial matrix production, and by formation of advanced glycation end products (AGEs), upregulation of heparanase expression, and activation of protein kinase C (19;21;22). The pathogenic effects of AGEs in diabetic nephropathy include glomerular hypertrophy, accumulation of matrix proteins, and proteinuria (23–25). Upregulation of heparanase results in loss of negative charged cell surface heparan sulfate and may contribute to increased GBM permeability to albumin (22;26). The role of protein kinase C in diabetes is mainly related to vascular complications (27). Furthermore, high glucose is a potent regulator of several growth factors, of which transforming growth factor (TGF)-β1, vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), connective tissue growth factor (CTGF), and bone morphogenetic protein (BMP)-7 have all been implicated in experimental and human diabetic nephropathy (28–32).

Hemodynamic factors relevant to the pathogenesis of diabetic nephropathy include systemic hypertension, intraglomerular hypertension, and vasoactive hormones, such as angiotensin II. For example, both proteinuria and structural glomerular injury are attenuated in diabetic rats after temporary blockade of the renin-angiotensin system (33). Of note, the contribution of angiotensin II to the development of diabetic nephropathy might also involve non-hemodynamic mechanisms, since it is known to be directly involved in accumulation of ECM components and in induction of profibrotic growth factors (34).

A novel aspect in the pathogenesis of diabetic nephropathy is the contribution of bone-marrow derived progenitor cells. For instance, non-diabetic mice developed albuminuria, glomerular hypertrophy, and ECM accumulation after receiving bone-marrow transplants from diabetic *db/db* mice (35). Circulating progenitor cells cultured from peripheral blood mononuclear cells comprise both endothelial progenitor cells (EPC) and myofibroblast progenitor cells (MFPC) (36). In patients with diabetes, dysfunction of circulating progenitor cells has been examined previously at the EPC level, which showed decreased outgrowth of diabetes-derived EPC along with impaired angiogenic activity (37;38).

Risk factors and biomarkers

Several factors have been identified in patients with diabetes that are associated with increased risk for developing diabetic nephropathy. Of the non-modifiable risk factors,

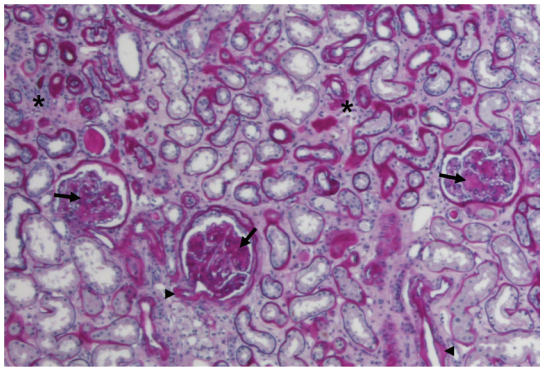

Figure 1. Histological changes of diabetic nephropathy. Diabetic nephropathy is characterized by diffuse and nodular glomerulosclerosis (arrows), hyaline arteriolosclerosis (arrow heads), and tubulointerstitial fibrosis (asterisks).

ethnicity, genetic susceptibility, and age were significantly associated with diabetic nephropathy. As for type 2 diabetes, the incidence and severity of diabetic nephropathy is increased in African-Americans, Mexican-Americans, Pima Indians, and Indo-Asians (39–42). The risk of African-Americans remained significantly increased after adjustment for higher prevalence of diabetes, socioeconomic status, and access to health care (39). Furthermore, the likelihood of developing both type 1 and type 2 diabetic nephropathy is markedly increased in patients with a diabetic sibling or parent who has diabetic nephropathy (43). Genetic factors associated with susceptibility to diabetic nephropathy include also polymorphisms in the genes encoding angiotensin converting enzyme, angiotensin-II type 2 receptor, aldose reductase, engulfment and cell motility 1, VEGF, adiponectin, and carnosinase 1 (44–50).

Modifiable risk factors associated with development of diabetic nephropathy are hypertension, hyperfiltration, and hyperglycemia. Several clinical studies have demonstrated that diabetic patients who develop diabetic nephropathy are more likely to have higher hemoglobin A1c values and higher blood pressure (2;51;52). The contribution of hyperfiltration to increased risk of diabetic nephropathy is evident from studies in which diabetic patients with initial high GFR appeared to have a higher risk for developing diabetic renal disease (53;54). Other modifiable risk factors associated with development of diabetic nephropathy are body mass index, smoking, and the use of oral contraceptives (55–57).

To date, UAE is the most important biomarker to predict development and progression of diabetic nephropathy. Changes in microalbuminuria were shown to be associated already with early decline in renal function in diabetic patients without diabetic nephropathy (58). In patients with overt diabetic nephropathy, levels of macroalbuminuria were strongly associated with increased rate of decline in GFR and progression to ESRD (59–61). In addition, soluble growth factors might be potential biomarkers in patients with diabetes. For instance, levels of TGF-β1 and VEGF in plasma and urine were elevated in patients with diabetes and were associated with diabetic nephropathy (62–64). In contrast, urinary excretion of epidermal growth factor (EGF) was reduced in patients with diabetes and correlated inversely with UAE (65;66). As for progression to mortality, elevated plasma levels of N-terminal pro-brain natriuretic peptide, placental growth factor, and asymmetric dimethylarginine have all been shown to be independent predictors of cardiovascular and overall mortality in patients with diabetic nephropathy (67–69).

Connective Tissue Growth Factor

CCN family

CTGF is a member of the CCN (CTGF/Cyr61/Nov) family, and is also known as CCN-2 (70). The CCN family consists of six matricellular regulatory proteins, which act by binding to extracellular signaling molecules via their distinct interaction domains. Members of the CCN family show a high degree of amino acid sequence homology and each member contains at least 38 conserved cysteine residues (71). CCN proteins demonstrate a high variety of biological functions, mainly dependent on the cellular context (72).

CTGF was first identified in conditioned media of endothelial cells as a 36–38 kDa cysteine-rich polypeptide containing chemotactic and mitogenic activity towards fibroblasts (73). Subsequently, CTGF was acknowledged as a key factor in ECM production and other profibrotic activity mediated by TGF-β1 (74). Resembling other members of the CCN family, biological functions of CTGF also include angiogenesis, chondrogenesis, osteogenesis, and control of cell adhesion, migration, proliferation and differentiation (72). CTGF exerts its biological activities by binding to e.g. fibronectin, extracellular signaling molecules, and to integrins (Figure 2).

CTGF in diabetic nephropathy

CTGF has been associated with numerous fibrotic disorders, but it is of particular interest to diabetic nephropathy. Soon after its identification, the expression of CTGF mRNA was shown to be strongly upregulated in human mesangial cells cultured under high glucose and in renal biopsies of patients with diabetic nephropathy (31;75). These observations were extended in rodent models of both type 1 and type 2 diabetic nephropathy (76–78).

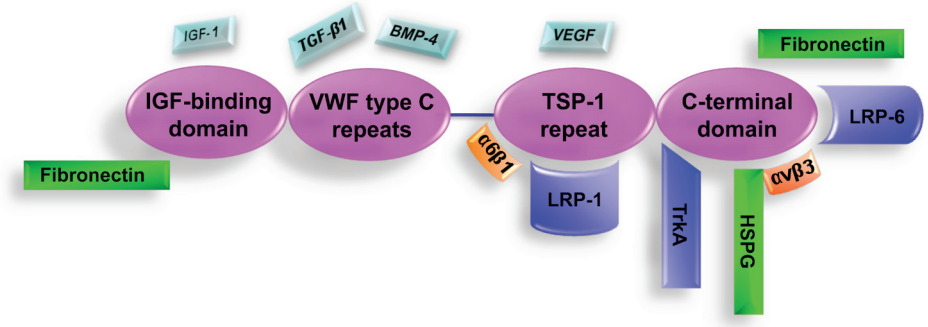

Figure 2. CTGF acts by binding to extracellular molecules via four distinct domains. Domain 1 consists of an N-terminal insulin-like growth factor (IGF)-binding protein, which is involved in binding of CTGF to IGF-1 and fibronectin (110;111). Domain 2 of CTGF shares high homology with Von Willebrand Factor (VWF) type C repeats as well as with chordin, and is responsible for binding of CTGF to TGF-β1 and BMP-4 (112). Domain 3 of CTGF contains a thrombospondin (TSP)-1 repeat and is involved in binding to VEGF, as well as binding to low-density lipoprotein receptor-related protein (LRP)-1 and integrin α6β1 (113–115). Domain 4 is a C-terminal cysteine-rich domain. This domain is involved in binding of CTGF to heparan sulfate proteoglycans (HSPG), integrin αvβ3, fibronectin, and to the Wnt co-receptor LRP-6 (111;116–118). Domain 4 is presumed to also interact with to the neurotrophin receptor TrkA (119).

Furthermore, severity of diabetic nephropathy was aggravated in transgenic mice with specific overexpression of CTGF in podocytes (79). In renal biopsies of patients with type 2 diabetic nephropathy, tubular expression of CTGF correlated with proteinuria, serum creatinine, and interstitial fibrosis (80). Upregulation of CTGF was also observed in the diabetic myocardium, liver, retina, and aorta (78;81–83). Moreover, treatment with CTGF antisense oligodeoxynucleotides proved to be successful in attenuation of proteinuria, and in reduction of genes involved in mesangial matrix expansion in mouse models of type 1 and type 2 diabetic nephropathy (84).

CTGF in biological fluids

CTGF contains an N-terminal signal peptide and is a secreted protein. By Western blot analysis, soluble CTGF has been detected in pig uterine secretory fluids as low mass forms between 10 and 20 kDa (85). Furthermore, 18, 24, and 38 kDa isoforms of CTGF have been identified in normal human serum and in amniotic, follicular, peritoneal, and cerebrospinal fluids (86).

The first observations of increased circulating CTGF levels in disease were reported in patients with biliary atresia and systemic sclerosis. By sandwich enzyme-linked immunosorbent assay, serum levels of CTGF were shown to be increased in patients with biliary atresia and systemic sclerosis. Moreover, circulating CTGF levels correlated with the progression of hepatic fibrosis in patients with biliary atresia and were associated with the extent of skin sclerosis and the severity of pulmonary fibrosis in patients with systemic sclerosis (87–89). In addition, CTGF levels were elevated in the vitreous of patients with diabetic retinopathy, and correlated with the degree of fibrosis in various vitreoretinal disorders (90;91). CTGF was also detected in peritoneal fluid of patients undergoing peritoneal dialysis, and was markedly elevated during episodes of peritonitis (92). In renal transplantation recipients, CTGF levels in serum and urine were increased, and urinary CTGF excretion correlated with the histological presence of chronic allograft nephropathy (93). Serum CTGF was also elevated in patients with fibrotic chronic inflammatory liver disease (94). In contrast, in patients with multiple myeloma and involvement of bone disease, serum CTGF was shown to be decreased as compared to normal subjects (95).

Soluble CTGF levels have also been determined in patients with diabetic nephropathy. CTGF levels were markedly increased in both plasma and urine of patients with diabetic nephropathy, but not in diabetic patients without nephropathy (96–99). However, these studies addressed too small numbers of patients to draw conclusions on the potential use of CTGF as biomarker for renal disease in patients with diabetes. When the studies described in this thesis were performed, no follow-up data were available regarding possible predictive value of plasma or urinary CTGF for outcome and disease progression.

Bone Morphogenetic Proteins

TGF-β superfamily

BMPs are members of the TGF-β superfamily, which consists of more than thirty growth factors including inhibins, activins, and the three isoforms of TGF-β. Comprising over twenty members, BMPs represent the largest subgroup of this family (100). BMPs are essential in development, throughout which they regulate cell growth, survival and differentiation, morphogenesis, and general organogenesis. On the basis of sequence and functional similarities with Drosophila family members, vertebrate BMPs have been traditionally divided into two subgroups. Vertebrate BMP-2 and BMP-4 are orthologues of *Drosophila decapentaplegic* and constitute the *dpp* subgroup. Vertebrate BMP-5, -6, -7, and -8 show high sequence similarity to the *Drosophila* gene glass bottom boat-60A and belong to the *gbb* subgroup. Although the wide phylogenetic distance between these two subgroups suggests that each group exhibits unique biological functions, it was recently demonstrated that, during kidney development, both BMP-6 and BMP-4 can substitute for loss of BMP-7 (101).

Members of the TGF-β superfamily signal through binding to type I and type II serine/threonine kinase receptors. Upon ligand binding and receptor complex activation, canonical signaling occurs through phosphorylation of receptor associated (R)-Smads. Phosphorylated R-Smads bind to their common partner Smad4, and translocate into the nuclei where they act as transcription factors. The specificity of the signal is mainly determined by the type I receptor, also known as activin receptor-like kinase (ALK), and the R-Smads. All BMPs bind ALK3 and -6, and activate Smad1, -5, and -8, while BMP-6 and BMP-7 can also signal through ALK2. Typically, TGF-βs bind ALK5 and activate Smad2 and -3, thus initiating profibrotic signaling. However, in the presence of L-endoglin, TGF-βs can also signal through ALK1, which activates Smad1, -5, and -8, and BMP target gene transcription. In the kidney, downstream targets of BMP include Id1, Id2, Id3, and Smad6, while TGF-β signaling through ALK5 induces CTGF, Smad7, and plasminogen activator inhibitor-1.

BMPs in the kidney

Several BMPs are expressed during kidney development, but only BMP-7 is absolutely required for proper formation of the kidney (102). In the adult mouse kidney, BMP-7 is expressed in glomerular podocytes, the thick ascending limb, the distal convoluted tubule, and most strongly in the collecting duct (103). Moreover, administration of BMP-7 results in striking improvement of renal function and structure in several experimental models of renal injury (104).

Although other BMPs are also expressed in the adult kidney, little is known about their function. Interestingly, comparative analysis of a number of members of the TGF-β superfamily identified BMP-6 as the most effective repressor of proinflammatory cytokines, which might indicate an important role also for BMP-6 in attenuation and treatment of renal injury (103).

Using transgenic reporter mice expressing enhanced green fluorescent protein (EGFP) under control of a BMP-responsive element (BRE) derived from the Id1 promoter, it

is possible to visualize spatiotemporal distribution of canonical BMP signaling activity by monitoring EGFP intensity. Consequently, direct immunofluorescence of renal tissue from these BRE-EGFP mice showed that BMP signaling activity in the kidney is mainly present in the podocytes and in the distal nephron (Figure 3).

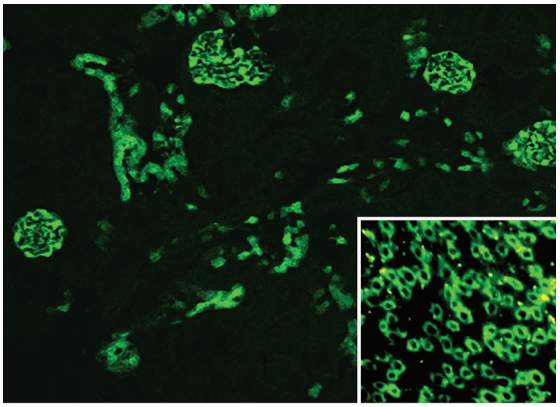

Figure 3. Distribution of BMP signaling in the kidney. BMP signaling activity, as demonstrated by direct immunofluorescence in renal tissue of BRE-EGFP mice, is mainly present in glomerular podocytes and in the papilla (inset).

BMP-7 in diabetic nephropathy

Of all BMPs, only BMP-7 has been described in relation to diabetic nephropathy. In diabetic rats, renal expression of BMP-7 was decreased by more than 90% (32). Decrease of BMP-7 expression was also observed in mouse podocytes cultured under high glucose and in renal biopsies of patients with diabetic nephropathy (105;106). The therapeutic potential of BMP-7 was demonstrated in rodent models of diabetic nephropathy, in which BMP-7 therapy attenuated both glomerular and tubulointerstitial damage (107;108). Furthermore, in diabetic transgenic mice overexpressing BMP-7 in podocytes and proximal tubuli, the severity of glomerulosclerosis, interstitial fibrosis, and albuminuria were all markedly decreased (109).

Aim and Outline of the Thesis

Diabetic nephropathy has a major impact on life expectancy and quality of life. The natural course of diabetic nephropathy is unpredictable and the pathogenesis of progression is not completely understood. To improve the identification and management of patients with diabetic nephropathy, novel biomarkers and better understanding of the disease process are essential.
Recently, CTGF and BMPs have emerged as key players in experimental and human renal fibrosis. The aim of the studies presented in this thesis is to elucidate the role of CTGF and BMPs, and their possible interaction in diabetic nephropathy.

CTGF is a secreted protein and can be detected in biological fluids. This renders CTGF of great interest for application as biomarker in patients with renal disease. The aim of **chapter II** was to measure urinary CTGF excretion in a cross-sectional study of patients with type 1 diabetes, and to determine the association of urinary CTGF excretion with diabetic nephropathy and clinical markers of renal disease. In **chapter III**, we evaluated the predictive value of plasma CTGF for end-stage renal disease and mortality in a prospective study of patients with type 1 diabetic nephropathy. **Chapter IV** describes the prediction of urinary CTGF excretion for deterioration of renal function in patients with idiopathic membranous nephropathy, which is one of the most frequent primary glomerular diseases in non-diabetic patients.

CTGF is a matricellular protein and exerts its biological effects mainly by modulating the activity of other growth factors. In **chapter V**, we investigated the role of CTGF as inhibitor of BMP-7 activity in experimental diabetic nephropathy and in cultured renal cells.

Bone marrow-derived circulating progenitor cells are important contributors to repair of damaged tissue and subject to regulation by growth factors. In **chapter VI**, we determined the outgrowth of myofibroblast progenitor cells in patients with type 1 diabetes and analyzed the downregulation of BMP-6 expression in these cells. The impact of BMP-6 deficiency on myofibroblast progenitor cells and renal response to injury was investigated in **chapter VII**.

Chapter VIII of this thesis reviews the current knowledge of BMP-7 and CTGF in the kidney, and discusses their therapeutic potential in renal fibrosis.

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Chapter II

Urinary CTGF Excretion Correlates with Clinical Markers of Renal Disease in a Large Population of Type 1 Diabetic Patients with Diabetic Nephropathy

TQ Nguyen[1], L Tarnow[2], S Andersen[2], P Hovind[2], HH Parving[2], R Goldschmeding[1], and FA van Nieuwenhoven[1]

[1]Department of Pathology, University Medical Center Utrecht, The Netherlands
[2]Steno Diabetes Center, Denmark

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Abstract

Objective: Levels of connective tissue growth factor (CTGF; CCN-2) in plasma are increased in various fibrotic disorders, including diabetic nephropathy. Recently, several articles have reported a strong increase of urinary CTGF excretion (U-CTGF) in patients with diabetic nephropathy. However, these studies addressed too small a number of patients to allow general conclusions to be drawn. Therefore, we evaluated U-CTGF in a large cross-sectional study of patients with type 1 diabetes.

Research design and methods: Subjects were 318 type 1 diabetic patients and 29 normoglycemic control subjects. U-CTGF was measured by sandwich enzyme-linked immunosorbent assay. Groups were compared by Kruskal-Wallis and Mann-Whitney analysis. The relation between U-CTGF and markers of diabetic nephropathy was determined by regression analysis.

Results: U-CTGF in patients with diabetic nephropathy (n=89, median 155 pmol/24 h [interquartile range 96–258]) was significantly higher than in microalbuminuric (n=79, 100 [65–78]) and normoalbuminuric (n=150, 85 [48–127]) patients and control subjects (n=29, 100 [78–114]). U-CTGF correlated with urinary albumin excretion (UAE) (R=0.31) and glomerular filtration rate (R=−0.38) in patients with diabetic nephropathy. A standardized increase in U-CTGF was associated with diabetic nephropathy (odds ratio 2.3 [95% CI 1.7–3.1]), which was comparable with the odds ratios for diabetic nephropathy of increased HbA1c (2.0 [1.5–2.7]), and blood pressure (2.0 [1.5–2.6]).

Conclusions: This is the first large cross-sectional study addressing U-CTGF in human type 1 diabetes. The observed association of U-CTGF with UAE and glomerular filtration rate might reflect a role of CTGF as progression promoter in diabetic nephropathy.

Introduction

Connective tissue growth factor (CTGF; CCN-2) is a 349 amino acid cysteine-rich polypeptide belonging to the CCN (CTGF/Cyr61/Nov) family (1). The CCN family consists of six matricellular regulatory proteins, which participate in diverse biological processes like angiogenesis and wound healing, and are involved in the control of cell proliferation and differentiation (2). *In vitro* studies have shown that CTGF is mainly involved in extracellular matrix synthesis and fibrosis. Upregulation of CTGF mRNA and increase of CTGF protein levels have been observed in various diseases, including diabetic nephropathy and cardiomyopathy, fibrotic skin disorders, systemic sclerosis, biliary atresia, liver fibrosis and idiopathic pulmonary fibrosis, and non-diabetic acute and progressive glomerular and tubulointerstitial lesions of the kidney (3–8). Furthermore, renal CTGF gene expression is strongly upregulated in experimental diabetic nephropathy (9;10).

CTGF is considered to be of particular interest to diabetic nephropathy because its expression is induced by high glucose and because it mediates transforming growth factor-β-dependent and -independent fibrogenesis (11;12). Recently, we have reported that plasma CTGF is increased in type 1 diabetic patients with diabetic nephropathy and that plasma CTGF levels in diabetic patients correlate with urinary albumin excretion (UAE) and glycemic control (13). Furthermore, urinary CTGF excretion (U-CTGF) is also increased in patients with diabetic nephropathy, and this is reduced by treatment with ACE inhibitors or angiotensin II receptor blockers (ARBs) (14–16). However, the latter studies were performed in relatively small groups of patients. Therefore, we set out to analyze in a large population of type 1 diabetic patients how U-CTGF levels relate to clinical parameters associated with (severity of) diabetic nephropathy.

Research Design and Methods

Subjects

For the present study, CTGF levels were determined in 24-h urine collections from 318 well-characterized adult type 1 diabetic patients and 29 healthy normoglycemic control subjects. Patients were selected from the outpatient clinic at Steno Diabetes Center (Gentofte, Denmark) for a study of diabetic nephropathy and proliferative retinopathy. Forty-three of 89 patients with diabetic nephropathy had been previously analyzed in a longitudinal study examining the impact of ARBs on U-CTGF (16).

Diabetic patients were categorized as having normoalbuminuria when UAE was persistently <30 mg/24 h, and type 1 diabetes was diagnosed at least 10 years ago. Patients were categorized as having microalbuminuria when UAE was between 30 and 300 mg/24 h in urine collections of at least two of three consecutive visits to the outpatient clinic. Patients were categorized as having diabetic nephropathy if they had persistent albuminuria (>300 mg/24 h) and diabetic retinopathy and no other kidney or renal tract disease. Demographic and clinical data were recorded, including age, sex, duration of diabetes, weight, height, and medication. Blood pressure values were measured twice with a Hawksley sphygmomanometer after 10 min of supine rest. Presence of retinopathy

was scored as nil, simplex, or proliferative by fundus photography. UAE was determined in 24-h urine collections by turbidimetry. In venous blood samples, plasma creatinine was determined (Cobas Mira Plus; Roche), and HbA1c was measured by high-performance liquid chromatography (normal range 4.1–6.4%) (Variant; Biorad Laboratories). Estimated glomerular filtration rate (GFR) was calculated by the Modification of Diet in Renal Disease study equation (17). The study was performed according to the principles of the Declaration of Helsinki and approved by the ethical committee of Copenhagen County. All patients and control subjects gave their informed consent.

Enzyme-linked immunosorbent assay for U-CTGF

U-CTGF was determined by a sandwich enzyme-linked immunosorbent assay, using monoclonal antibodies against two distinct epitopes on the NH2-terminal part of human CTGF (FibroGen, South San Francisco, CA), as described earlier (13). Briefly, microtiter plates were coated overnight with capture antibody and blocked with BSA. Urine samples were diluted three-fold in assay buffer, and a 50-µl diluted sample was added to each well together with 50-µl CTGF detection antibody conjugated with alkaline phosphatase. Plates were washed and a substrate solution containing p-nitrophenyl phosphate was added to each well. Absorbance was read at 405 nm. Purified recombinant human CTGF (FibroGen) was used for calibration. The detection limit of this assay was 4 pmol/l, and intra- and interassay variations were 6 and 20%, respectively. The assay detects both CTGF NH2-terminal fragments as well as full-length CTGF. To avoid confusion due to differences in molecular mass of full-length CTGF and fragments, U-CTGF is expressed as pmol/24 h.

Statistical analysis

Data are expressed as means±SD, unless otherwise stated. UAE, plasma creatinine, and U-CTGF were logarithmically transformed before analysis because of their skewed distribution. Difference in U-CTGF between groups was determined by Mann-Whitney analysis or Kruskal-Wallis analysis followed by Dunn's method. Pearson or Spearman correlations were calculated between U-CTGF and clinical markers of diabetic nephropathy. Forward stepwise regression analysis was used to compare CTGF levels with relevant patient characteristics and clinical parameters. Logistic regression analysis was used to identify parameters that are associated with diabetic nephropathy. Odds ratios for continuous variables were standardized for 1-SD difference. In all cases, $P<0.05$ was considered significant (two-tailed).

Results

U-CTGF excretion is increased in patients with diabetic nephropathy

General characteristics and clinical parameters of healthy subjects and diabetic patients are summarized in Table 1. A significant difference in U-CTGF was observed between patients with diabetic nephropathy and patients with microalbuminuria, between patients with diabetic nephropathy and patients with normoalbuminuria, and between patients with diabetic nephropathy and healthy control subjects ($P<0.01$).

Table 1. General and clinical parameters of healthy control subjects and type 1 diabetic patients

		Type 1 diabetic patients		
	Control subjects	**Normoalbuminuria**	**Microalbuminuria**	**Diabetic nephropathy**
General patient characteristics				
N (% men)	29 (66)	150 (48)	79 (48)	89 (48)
Age (years)	42 ± 9*†	53 ± 13‡§	53 ± 12‡§	45 ± 10*†
Duration of diabetes (years)	–	34 ± 11	37 ± 10§	32 ± 10†
BMI (kg/m^2)	24.2 ± 2.4	24.4 ± 4.7	24.8 ± 3.3	25.2 ± 3.4
Retinopathy (nil/simplex/proliferative)	–	52/1/97	8/2/69	0/10/79
Antihypertensive treatment (ACEi/ARB/other)	0/0/0	24/4/58	47/9/32	20/15/24#
Glycemic control				
Blood glucose (mmol/l)	4.6 ± 0.6*†§	9.4 ± 4.4‡	9.1 ± 4.5‡	8.9 ± 4.7‡
HbA1c (%)	5.4 ± 0.3*†§	8.3 ± 1.1†‡§	8.8 ± 0.9*‡§	8.9 ± 1.2*‡
Diabetic nephropathy				
Urinary albumin excretion (mg/24h)	5 (4–7)†§	6 (4–11)†§	36 (17–68)*‡	661 (251–1495)*†‡
Plasma creatinine (μmol/l)	90 (78–102)§	83 (74–92)§	89 (80–100)§	97 (84135)*†‡
Estimated GFR (ml/min per 1.73 m^2)	80 ± 13§	79 ± 15§	73 ± 18§	63 ± 25*†‡
Systolic blood pressure (mm Hg)	123 ± 10*†§	140 ± 20‡	145 ± 20‡	145 ± 19‡
Diastolic blood pressure (mm Hg)	74 ± 6	74 ± 9	74 ± 9	81 ± 12*†‡
CTGF levels				
Urinary CTGF excretion (pmol/24 h)	100 (78–114)§	85 (48–127)§	100 (65–78)§	155 (96–258)*†‡

Data are means±SD or median (interquartile range), unless otherwise indicated. #In the framework of participation in another study, 43 of 89 patients with diabetic nephropathy had stopped antihypertensive medication 4 weeks prior to sample collection (16). *P<0.05 vs. normoalbuminuria; †P<0.05 vs. microalbuminuria; ‡P<0.05 vs. control subjects; §P<0.05 vs. diabetic nephropathy.

Mean U-CTGF was not significantly different between 20 patients with diabetic nephropathy who received ACE inhibition at the time of sample collection and 69 patients with diabetic nephropathy who were not treated with ACE inhibition (194 in treated vs. 146 pmol/24 h in untreated patients, P=0.27). Of 69 patients without ACE inhibitors, 26 had never used this medication, while 43 had stopped antihypertensive medication 4 weeks before sample collection in the framework of participation in another longitudinal study examining the impact of ARBs on U-CTGF (16).
No significant difference was observed in U-CTGF between diabetic patients with or without simplex or proliferative retinopathy (P=0.34, data not shown). In the subgroup of normoalbuminuric patients, U-CTGF was slightly lower in patients with retinopathy compared with patients without retinopathy (61.6 vs. 85.1 pmol/24 h, respectively, P<0.05).

U-CTGF correlates with albuminuria and with declined renal function
U-CTGF correlated with UAE in all patients (R=0.43, P<0.01) and also in the subgroups of normoalbuminuric (R=0.29, P<0.01) and microalbuminuric (R=0.25, P<0.01) patients and patients with diabetic nephropathy (R=0.31, P<0.01, Figure 1A).
Since GFR was not measured in all patients, we estimated GFR using the Modification of Diet in Renal Disease study method described by Levey *et al*. (17). U-CTGF inversely correlated with estimated GFR in all patients (R=−0.26, P<0.01). When subgroups of patients were examined separately, this correlation was the strongest in patients with diabetic nephropathy (R=−0.37, P<0.01, Figure 1B) and also present in microalbuminuric patients (R=−0.26, P<0.05) but not in normoalbuminuric patients (R=0.14, P=0.08).

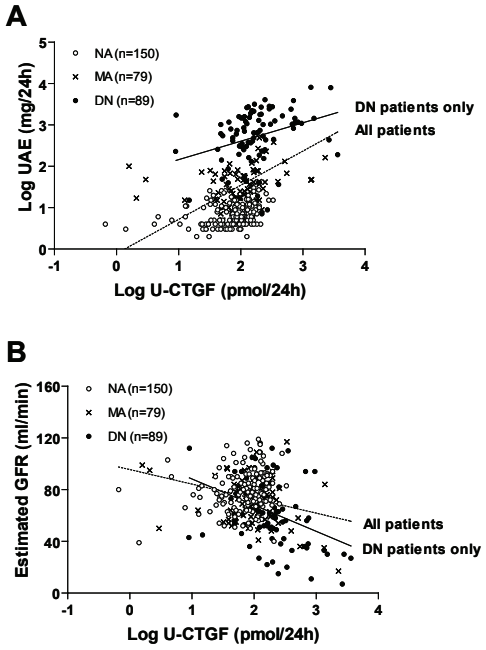

Figure 1. Correlations between U-CTGF and clinical markers of renal disease. A: Log-transformed U-CTGF correlates with log-transformed UAE in all patients (dashed line, R=0.43, P<0.01) and in patients with diabetic nephropathy (solid line, R=0.31, P<0.01). B: Log U-CTGF inversely correlates with estimated GFR in all patients (dashed line, R=−0.26, P<0.001), and this correlation is the strongest in patients with diabetic nephropathy (solid line, R=−0.37, P<0.001). DN, diabetic nephropathy; MA, microalbuminuria; NA, normoalbuminuria.

Associations between U-CTGF, diabetic nephropathy, and clinical parameters
Forward stepwise regression analysis was performed to examine associations of U-CTGF with relevant clinical parameters (i.e., sex, age, duration of diabetes, BMI, HbA1c, UAE, GFR, systolic and diastolic blood pressure, presence of retinopathy, and use of ACE inhibitors or ARBs). Parameters with P<0.1, as determined by Pearson or Spearman correlations, were sex, diastolic blood pressure, UAE, and GFR (Table 2).

Table 2. Correlations of log-transformed U-CTGF with clinical parameters

Parameter	R	P value
Sex	0.178	<0.001
Age	–0.050	0.357
Duration of diabetes	0.038	0.494
BMI	0.035	0.511
HbA1C	0.064	0.235
UAE	0.419	<0.001
GFR	–0.243	<0.001
Systolic blood pressure	0.061	0.254
Diastolic blood pressure	0.094	0.080
Presence of retinopathy*	0.011	0.847
ACE inhibitors*	0.070	0.224
ARBs*	0.058	0.311

Correlations were determined by calculation of Pearson or *Spearman R.

These parameters were subsequently entered into forward stepwise regression analysis with U-CTGF as a dependent variable. In all patients, U-CTGF correlated most strongly with UAE (R=0.43), whereas UAE and GFR together resulted in a cumulative R of 0.45. Correlation of U-CTGF with UAE, GFR, and sex together yielded a cumulative R of 0.48. Addition of other parameters did not significantly contribute to the correlation. Within the subgroup of patients with diabetic nephropathy, U-CTGF correlated most strongly with GFR (R=0.37), whereas GFR and UAE together yielded a cumulative R of 0.47. In normoalbuminuric and microalbuminuric patients, U-CTGF correlated most strongly with UAE (R=0.29) and GFR (R=0.26), respectively (Table 3).
When in patients with diabetic nephropathy, instead of U-CTGF, UAE or GFR was taken as a dependent variable in the forward stepwise regression analysis of this dataset, U-CTGF was identified as the strongest independent predictor of these parameters (R=0.31 and 0.36, respectively). It thus appears that in patients with diabetic nephropathy, U-CTGF is correlated with the two most important clinical markers for severity of renal disease.

Logistic regression analysis was performed to further assess the association of U-CTGF and other parameters with diabetic nephropathy. The parameters used in this analysis were U-CTGF, sex, HbA1C, BMI, and blood pressure. A standardized (1-SD) increase

in U-CTGF was associated with diabetic nephropathy (odds ratio 2.3 [95% CI 1.7–3.1]), which was comparable with the odds ratios for diabetic nephropathy of increased HbA1c (2.0 [1.5–2.7]) and blood pressure (2.0 [1.5–2.6]).

Table 3. Forward stepwise regression analysis of log-transformed U-CTGF with predictors in all patients and the subgroups of diabetic patients

Parameter	Cumulative R	P value
All patients (n=347)		
UAE	0.430	<0.001
GFR	0.451	<0.001
Sex	0.481	<0.001
Normoalbuminuria (n=150)		
UAE	0.291	<0.001
Sex	0.334	<0.001
Diastolic blood pressure	0.375	<0.001
Microalbuminuria (n=79)		
GFR	0.262	0.019
Sex	0.371	0.004
Diabetic nephropathy (n=89)		
GFR	0.371	<0.001
UAE	0.468	<0.001

Correlations were determined by calculation of Pearson or *Spearman R.

Conclusions

In the present study, we analyzed U-CTGF excretion in 318 well-characterized patients with type 1 diabetes and 29 healthy control subjects. Thus far, three much smaller studies have been published that addressed elevated CTGF levels in urine of patients with diabetic nephropathy (14–16). Our results, which were obtained in a large population of type 1 diabetic patients, confirm that U-CTGF is significantly increased in diabetic nephropathy. To this, we add that in patients with diabetic nephropathy, U-CTGF is correlated with UAE ($R=0.31$, $P<0.01$) and GFR ($R=-0.37$, $P<0.01$), both important clinical markers for severity of renal disease. Although these R values are relatively small, logistic regression analysis revealed that the association of U-CTGF with diabetic nephropathy was comparable with that of the established risk factors HbA1c and blood pressure. The low R values of individual parameters most likely relate to the multifactorial pathogenesis of diabetic nephropathy, which, in addition to the above risk factors, also includes genetic susceptibility.

Although individual levels ranged up to 37 times the mean level in normal control subjects, mean U-CTGF in patients with diabetic nephropathy was only 1.6-fold higher than in healthy control subjects, and there was extensive overlap between the patient and control groups. Although statistically significant, the observed differences thus appear less impressive than those reported in previous, much smaller studies.
In a preliminary report, Riser *et al.* (14) observed an approximate six-fold increase of U-CTGF in seven diabetic patients with renal disease compared with six healthy volunteers. However, because of different methodological approach, comparison with our study is difficult.
In the study by Gilbert *et al.* (15), U-CTGF level was determined using a sandwich enzyme-linked immunosorbent assay with the same antibodies and standard as applied in the present study. In the former study, 8 untreated (i.e., without ACE inhibition) diabetic patients with microalbuminuria and 5 patients with macroalbuminuria had U-CTGF levels 10- and 100-fold higher than 10 diabetic patients with normoalbuminuria. However, the mean age of the five untreated diabetic patients with macroalbuminuria in this study was 24 years higher, and mean duration of diabetes in these patients was 21 years longer than that of diabetic patients with normoalbuminuria. It thus appears that within the population of diabetic nephropathy, there are huge differences in U-CTGF between individual patients but that mean U-CTGF is only moderately increased.

As for the effect of antihypertensive treatment on U-CTGF level, the study cited above (15) also reported that U-CTGF of an additional eight patients with diabetic nephropathy who received ACE inhibition was 30-fold lower compared with the U-CTGF of those without ACE inhibition. However, as discussed already in that report, the patients in the untreated group were older and had longer duration of diabetes, as well as worse renal function than the treated patients. In the present study, no effect of ACE inhibition on U-CTGF was observed, but the untreated patients with diabetic nephropathy had slightly better GFR than the treated patients. After adjustment for differences in renal function, U-CTGF levels in the present study are comparable with those of Gilbert *et al.* (15).
Although the direct impact of ACE inhibition on U-CTGF thus might seem to be limited, conclusions regarding the impact of ACE inhibitors and ARBs compared with conventional antihypertensive treatment or no blood pressure-lowering therapy should not be drawn from this cross-sectional study because of confounding by indication (18), i.e., the most severely affected patients are likely to have received treatment that differs from that of less severely affected patients. Irrespective of this, reported induction of CTGF by angiotensin II (19) and reduction of U-CTGF following angiotensin II receptor blockade (16) do clearly imply involvement of the renin-angiotensin-aldosterone system in regulation of U-CTGF.

Diabetic retinopathy is closely associated with diabetic nephropathy. Diabetic retinopathy is much more frequent and often precedes the onset of diabetic nephropathy (20). Patients with diabetic retinopathy have increased CTGF in the vitreous and microvascular pericytes of the retina (21;22). Since diabetic retinopathy might be a reflection of systemic microvascular damage (23), its presence and severity might influence plasma CTGF, which would also be reflected in the (filtered) CTGF level in urine. However,

U-CTGF levels in diabetic patients with retinopathy were not increased compared with those in patients without retinopathy. In normoalbuminuric patients, U-CTGF was even slightly lower in patients with diabetic retinopathy compared with patients without diabetic retinopathy, but this might relate to lower GFR in normoalbuminuric patients with diabetic retinopathy than in those without diabetic retinopathy (77 vs. 83 ml/min per 1.73 m^2, $P<0.05$).

It is still unclear how much plasma CTGF levels contribute to U-CTGF. From their physicochemical properties, CTGF and its fragments are predicted to be cleared from plasma by glomerular filtration. Plasma CTGF levels were not available for the patients in the present study. However, in other studies of human and experimental diabetes, we have observed an approximate two- to three-fold increase of plasma CTGF and two- to four-fold upregulation of CTGF mRNA expression in kidney, heart, and liver (13;24). These data suggest that both local production of CTGF in the kidney and renal filtration of (elevated) plasma CTGF might be involved in increased U-CTGF. In addition, tubular dysfunction and/or saturation of tubular reabsorption capacity in proteinuric patients with diabetic nephropathy might also contribute to higher levels of CTGF in voided urine.

In summary, the main finding of this study is that in patients with diabetic nephropathy, U-CTGF is elevated and correlates with severity of renal disease in terms of both UAE and decreased GFR. This suggests that CTGF, probably in conjunction with other factors, might act as a progression promoter in diabetic nephropathy. The possible pathogenic role of CTGF in (progression of) diabetic nephropathy is now the subject of studies in animal models, in which CTGF ligand availability is manipulated on a background of experimental diabetes (25;26). In addition, prognostic clinical studies will be necessary to evaluate U-CTGF as an additional parameter for monitoring of deterioration of renal function in diabetic nephropathy.

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Chapter III

Plasma CTGF is an Independent Predictor of End-Stage Renal Disease and Mortality in Type 1 Diabetic Nephropathy

TQ Nguyen[1], L Tarnow[2], A Jorsal[2], N Oliver[3], P Roestenberg[1], Y Ito[4], HH Parving[5], P Rossing[2], FA van Nieuwenhoven[1], and R Goldschmeding[1]

[1]Department of Pathology, University Medical Center Utrecht, The Netherlands
[2]Steno Diabetes Center, Gentofte, Denmark
[3]FibroGen, Inc., South San Francisco, CA, USA
[4]Department of Nephrology, Nagoya University School of Medicine, Japan
[5]Department of Endocrinology, Rigshospitalet, Copenhagen, Denmark

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Abstract

Objective: We evaluated the predictive value of baseline plasma connective tissue growth factor (CTGF; CCN-2) in a prospective study of patients with type 1 diabetes.

Research design and methods: Subjects were 198 type 1 diabetic patients with established diabetic nephropathy and 188 type 1 diabetic patients with persistent normoalbuminuria. Follow-up time was 12.8 years. Prediction of end-stage renal disease (ESRD) and mortality by plasma CTGF was analyzed in conjunction with conventional risk factors.

Results: Plasma CTGF was higher in patients with nephropathy than in patients with normoalbuminuria (median 381 [interquartile range 270–630] vs. 235 [168 –353] pmol/l). In patients with nephropathy, elevated plasma CTGF was an independent predictor of ESRD (covariate-adjusted hazard ratio [HR] 1.6 [95% CI 1.1–2.5]) and correlated with the rate of decline in glomerular filtration rate (GFR) (cumulative R=0.46). Area under the receiver operating characteristic curve for prediction of ESRD was 0.72. Plasma CTGF above a cutoff level of 413 pmol/l predicted ESRD with a sensitivity of 73% and a specificity of 63% and was associated with a higher rate of decline in GFR (5.4±4.9 vs. 3.3±3.5 ml/min per 1.73 m^2 per year). Moreover, in patients with nephrotic range albuminuria (3 g/day), plasma CTGF was the only predictor of ESRD (covariate-adjusted HR 4.5 [2.0–10.4]). Plasma CTGF was an independent predictor also of overall mortality (covariate-adjusted HR 1.4 [1.1–1.7]). In contrast, in normoalbuminuric patients, plasma CTGF did not correlate with clinical parameters and did not predict outcome.

Conclusions: Plasma CTGF contributes significantly to prediction of ESRD and mortality in patients with type 1 diabetic nephropathy.

Introduction

Diabetic nephropathy is the most important cause of end-stage renal disease (ESRD) and contributes importantly to mortality, mainly through increases in cardiovascular disease (1). However, the course of diabetic nephropathy remains unpredictable, and the pathogenesis of progression is not completely understood.

Connective tissue growth factor (CTGF; CCN-2) was first identified in conditioned media of endothelial cells as a 36- to 38-kDa polypeptide containing chemotactic activity toward fibroblasts (2). CTGF has been acknowledged as a key factor in extracellular matrix production and other profibrotic activity mediated by transforming growth factor-β1 (3). Other biological functions of CTGF include angiogenesis, chondrogenesis, osteogenesis, and cell adhesion, migration, proliferation, and differentiation (4).

Recently, CTGF has emerged as an important factor in diabetic nephropathy. In renal cells, CTGF is induced by high glucose, and it is critically involved in diabetes-associated changes like extracellular matrix synthesis, cell migration, and epithelial-to-mesenchymal transition (5–8). Furthermore, upregulation of CTGF has been observed in human and experimental diabetic nephropathy (6;9–12), whereas the structure and function of the kidney are largely preserved in diabetic mice with hemizygous CTGF deletion and in diabetic mice treated with CTGF antibody or CTGF antisense oligonucleotides (13–15).

CTGF is a secreted protein and can be detected in biological fluids. Previous small studies have reported that both urinary CTGF excretion and plasma CTGF levels are elevated in patients with diabetic nephropathy (16–19). Recently, we have shown in a large cross-sectional study of patients with type 1 diabetes that urinary CTGF excretion is associated with urinary albumin excretion (UAE) and inversely with glomerular filtration rate (GFR), both important clinical markers for severity of renal disease (20). In aggregate, these data suggest that CTGF might be a useful marker of renal deterioration in patients with diabetic nephropathy.

Because all previous clinical studies addressing CTGF as a biomarker were performed in cross-sectional designs, the possible prognostic value of CTGF levels in diabetic nephropathy still has remained elusive. Therefore, we set out to evaluate whether plasma CTGF might predict loss of GFR, ESRD, and mortality in a prospective study of 386 type 1 diabetic patients with and without diabetic nephropathy, during a follow-up period of 12.8 years.

Research Design and Methods

Subjects

Patients with type 1 diabetic nephropathy attending the outpatient clinic of Steno Diabetes Center in 1993 were invited to participate in a case-control study (21;22). Type 1 diabetes was considered present if the age at onset of diabetes was ≤35 years and time to definite insulin therapy was ≤1 year. Patients were categorized as having diabetic nephropathy if they had persistent albuminuria (>300 mg/day) in at least two of three consecutive 24-h urine collections, diabetic retinopathy, and no other kidney or renal tract disease (23). Patients with equally long-lasting type 1 diabetes (>15 years)

and persistent normoalbuminuria (<30 mg/day), who were matched for sex, age, and duration of diabetes, served as control subjects. Thus, 198 patients with nephropathy and 188 patients with normoalbuminuria were included in the study. The study was approved by the local ethics committee, in accordance with the Declaration of Helsinki, and all patients gave their informed written consent.

Investigations were performed in the morning after an overnight fast. Blood pressure was measured twice after at least 10 min rest in the supine position. UAE was measured by an enzyme immunoassay from 24-h urine collections. Plasma creatinine was assessed by a kinetic Jaffé method. GFR was measured after a single intravenous injection of ^{51}Cr-EDTA (3.7 MBq) by following the plasma clearance of the tracer for 4 h (24). Linear regression of yearly GFR measurements in each individual was used to estimate rate of decline in GFR. In normoalbuminuric patients, GFR was estimated by the Modification of Diet in Renal Disease equation (25). Diabetic retinopathy was assessed by fundus photography and graded as nil, simplex, or proliferative retinopathy. Patients were interviewed using the World Health Organization cardiovascular questionnaire. Major cardiovascular events were diagnosed as a history of stroke and/or myocardial infarction. Smoking was defined as smoking ≥1 cigarettes/cigars/pipes a day.

In a prospective observational study design, patients were followed up until 1 November 2006 or until death (n=99) or emigration (n=3). If a patient had died, the date of death was recorded, and the cause of death was obtained from the death certificate. Additional available information from necropsy reports was included. All deaths were classified as cardiovascular deaths unless an unequivocal noncardiovascular cause was established. Information about the date of ESRD was obtained from patient records or discharge letters from other hospitals. ESRD was defined as the need for dialysis or renal transplantation.

ELISA for plasma CTGF

CTGF was measured in plasma samples drawn at study entry that had been stored at –80˚C at the Steno Diabetes Center. Storage time and freeze-thaw cycles of all samples were identical. CTGF was determined by a sandwich ELISA using monoclonal antibodies against two distinct epitopes on the N-terminal part of human CTGF (FibroGen, South San Francisco, CA) as described previously (18;20).

Statistical Analysis

Normally distributed variables are expressed as means±SD. UAE, plasma creatinine, and plasma CTGF were logarithmically transformed before analysis and are expressed as medians (interquartile range). Comparisons between groups were performed by unpaired Student's *t*-test or Mann-Whitney test. Multiple logistic regression analysis was used to identify the contribution of parameters to risk of diabetic nephropathy. Pearson and Spearman correlations and forward stepwise regression analysis were used to identify parameters that correlated with rate of decline in GFR. All time-to-event variables were analyzed using log-rank tests and were displayed on Kaplan-Meier plots according to levels being above or below the cutoff value, as determined by the receiver operating characteristic (ROC) curve. The cutoff value with the most discriminative value was defined as the point of the ROC curve closest to the left upper corner ($d = \sqrt{[(1\text{-specificity})^2 + (1\text{-sensitivity})^2]}$).

Cox proportional hazard regression models with forward selection were used to evaluate the contribution of baseline covariates to ESRD and overall mortality. For this, continuous variables were standardized for one standard deviation (1-SD) difference. In the Cox regression model for ESRD, baseline covariates that were associated with rate of decline in GFR ($P<0.1$) were entered into the model. These covariates were plasma CTGF, sex, duration of diabetes, UAE, GFR, and systolic blood pressure. In the Cox regression models for cardiovascular and overall mortality, the following prespecified baseline covariates were entered into the model: plasma CTGF, sex, age, smoking, UAE, GFR, HbA1c, history of cardiovascular event, and systolic blood pressure.
Results are given as hazard ratios (HRs) with 95% confidence intervals. In all cases, $P<0.05$ was considered significant (two-tailed). All calculations were performed using SPSS (version 12.0; SPSS, Chicago, IL).

Results

Plasma CTGF is increased in patients with diabetic nephropathy
General characteristics and baseline parameters of patients are summarized in Table 1. Plasma CTGF was higher in patients with diabetic nephropathy than in patients with normoalbuminuria (median 381 [interquartile range 270–630] vs. 235 [168–353] pmol/l; $P<0.001$). In patients with nephropathy, plasma CTGF correlated with UAE ($R=0.16$, $P=0.02$), and inversely with GFR ($R=-0.58$, $P<0.001$) (Figure 1A and B). In these patients, plasma CTGF was higher in those receiving antihypertensive medication (n=151) compared with those not receiving antihypertensive medication (n=47) (588 [289–697] vs. 333 [197–422] pmol/l; $P=0.01$), but this difference disappeared after adjustment for GFR. In patients with normoalbuminuria, plasma CTGF did not correlate with any of the clinical parameters.

Plasma CTGF contributes to risk of diabetic nephropathy
After adjustment for duration of diabetes, BMI, systolic blood pressure, HbA1c, and GFR, a standardized increase of plasma CTGF resulted in 2.0-fold increased chance of diabetic nephropathy (odds ratio [OR] 2.0, [95% CI 1.5–2.8]). This result was comparable with the ORs for diabetic nephropathy of increased HbA1c (2.2 [1.6–2.9]) and systolic blood pressure (1.7 [1.5–2.9]) after adjustment for duration of diabetes, GFR, plasma CTGF, and systolic blood pressure or HbA1c, respectively.

Baseline plasma CTGF correlates with rate of decline in GFR
In patients with nephropathy, the mean rate of decline in GFR was 4.3 ml/min per 1.73 m^2 per year. With rate of decline in GFR as a dependent variable, UAE was identified as the regression parameter with the strongest correlation ($R=0.43$, $P<0.001$). Applying significance cutoff at $P<0.05$, baseline plasma CTGF was the next and only parameter significantly contributing to an increase in this correlation, resulting in a cumulative R of 0.46 ($P=0.001$). Addition of other parameters did not significantly increase correlation with rate of decline in GFR. In particular, the effect of plasma CTGF concentration on this regression was independent of baseline GFR.

Similarly, in separate analysis of Kidney Disease Outcomes Quality Initiative (K/DOQI) subgroups for chronic kidney disease, patients with plasma CTGF above the median of their particular subgroup tended to have a higher rate of decline in GFR, but this trend did not reach statistical significance (Figure 1C). Also within subgroups stratified for UAE, a similar trend of a higher rate of decline in GFR was observed in patients with higher plasma CTGF levels (Figure 1D).

Table 1. Baseline parameters of 386 type 1 diabetic patients with and without diabetic nephropathy

	Nephropathy	Normoalbuminuria	P value
General patient characteristics			
n (% men)	198 (62)	188 (61)	0.84
Age (years)	41.0 ± 9.5	42.5 ± 9.9	0.14
Duration of diabetes (years)	27.7 ± 8.0	26.8 ± 8.5	0.26
BMI (kg/m^2)	24.0 ± 3.3	23.7 ± 2.5	0.20
Retinopathy (nil/simplex/proliferative)	0/61/137	66/103/19	< 0.001
HbA1c (%)	9.6 ± 1.5	8.5 ± 1.1	< 0.001
Renal function			
UAE (mg/24 h)	794 (342–2050)	8 (5–13)	–
GFR (ml/min per 1.73 m^2)	74 ± 33	94 ± 16	< 0.001
Plasma creatinine (μmol/l)	103 (82–134)	76 (69–83)	< 0.001
Cardiovascular characteristics			
Systolic blood pressure (mm Hg)	151 ± 23	132 ± 18	< 0.001
Diastolic blood pressure (mm Hg)	86 ± 13	76 ± 10	< 0.001
Smokers	99 (50%)	81 (43%)	0.17
History of myocardial infarction	10 (5.1%)	2 (1.1%)	0.036
History of stroke	14 (7.1%)	1 (0.5%)	0.001
CTGF levels			
Plasma CTGF (pmol/l)	381 (270–630)	235 (168–353)	< 0.001

Data are means±SD or median (interquartile range).

Baseline plasma CTGF predicts ESRD in diabetic nephropathy

At baseline, 6 of 198 patients with diabetic nephropathy had already developed ESRD. These patients were excluded from further prospective analyses. Area under the ROC curve for prediction of ESRD by plasma CTGF was 0.72, for UAE was 0.73, and for systolic blood pressure was 0.68. The optimal cutoff value for plasma CTGF (413 pmol/l) predicted ESRD with a sensitivity of 73% and a specificity of 63% (Figure 1E).

During follow-up, 40 of 192 patients with nephropathy and none of the patients without nephropathy developed ESRD. Within the nephropathy group, development of ESRD

occurred in a larger proportion of patients with plasma CTGF >413 pmol/l (P<0.001) (Figure 1F). The rate of decline in GFR was higher in these patients than in those with plasma CTGF <413 pmol/l (5.4±4.9 vs. 3.3±3.5 ml/min per 1.73 m^2 per year, P<0.001) (Figure 1G).

In all patients with diabetic nephropathy, the covariate-adjusted HR of plasma CTGF for ESRD was 1.6 (95% CI 1.1–2.5, P=0.03). In patients with nephrotic range albuminuria (>3 g/day), 17 of 32 patients developed ESRD, consistent with previous studies in type 1 and type 2 diabetes (26;27). In this subgroup, plasma CTGF was the only independent predictor of ESRD, with a covariate-adjusted HR of 4.5 (2.0–10.4, P<0.001) (Table 2). In patients with non-nephrotic range albuminuria, the only independent predictor of ESRD was GFR (data not shown).

Table 2. Cox proportional hazard model for ESRD of baseline risk factors associated with rate of decline in GFR in patients with diabetic nephropathy

	Hazard ratio (95% CI)	P value
All patients with diabetic nephropathy (n=198)		
GFR (per 1-SD decrease = 34 ml/min per 1.73 m^2)	3.13 (1.90–5.15)	< 0.001
Sex (male vs. female)	2.52 (1.23–5.14)	0.01
UAE (per 1-SD increase = 3.6-fold)	2.08 (1.44–3.01)	< 0.001
Plasma CTGF (per 1-SD increase = 1.9-fold)	1.62 (1.05–2.50)	0.03
Patients with nephrotic range albuminuria (n=33)		
Plasma CTGF (per 1-SD increase = 1.9-fold)	4.53 (1.96–10.44)	< 0.001

Adjusted for systolic blood pressure, duration of diabetes, and all other variables of this table.

Baseline plasma CTGF predicts mortality in diabetic nephropathy

Mortality in patients with nephropathy was 40%, of which 54% was due to cardiovascular mortality. In patients with diabetic nephropathy, area under the ROC curve for prediction of both overall mortality and cardiovascular mortality by plasma CTGF was 0.66. A cutoff value for plasma CTGF of 413 pmol/l predicted overall mortality with optimal sensitivity and specificity (59% and 63%, respectively). A cutoff value for plasma CTGF of 419 pmol/l predicted cardiovascular mortality with optimal sensitivity and specificity (67% and 61%, respectively). Both cardiovascular mortality and overall mortality were higher in patients with plasma CTGF above either of these cutoff values (P<0.001) (overall mortality shown in Figure 1H).

Significant independent baseline predictors of overall mortality in patients with nephropathy were sex, plasma CTGF, HbA1c, systolic blood pressure, and age. The covariate-adjusted HR of plasma CTGF for prediction of overall mortality was 1.4 (95% CI 1.1–1.7) (P=0.005) (Table 3). In the subpopulation of nephrotic range albuminuric patients, this HR was 2.3 (1.2–4.4) (P=0.01). As for cardiovascular mortality, this was predicted independently by history of major cardiovascular event, systolic blood pressure, and GFR, but not by plasma CTGF.

In normoalbuminuric patients, overall mortality was 11%, of which 42% was due to cardio-

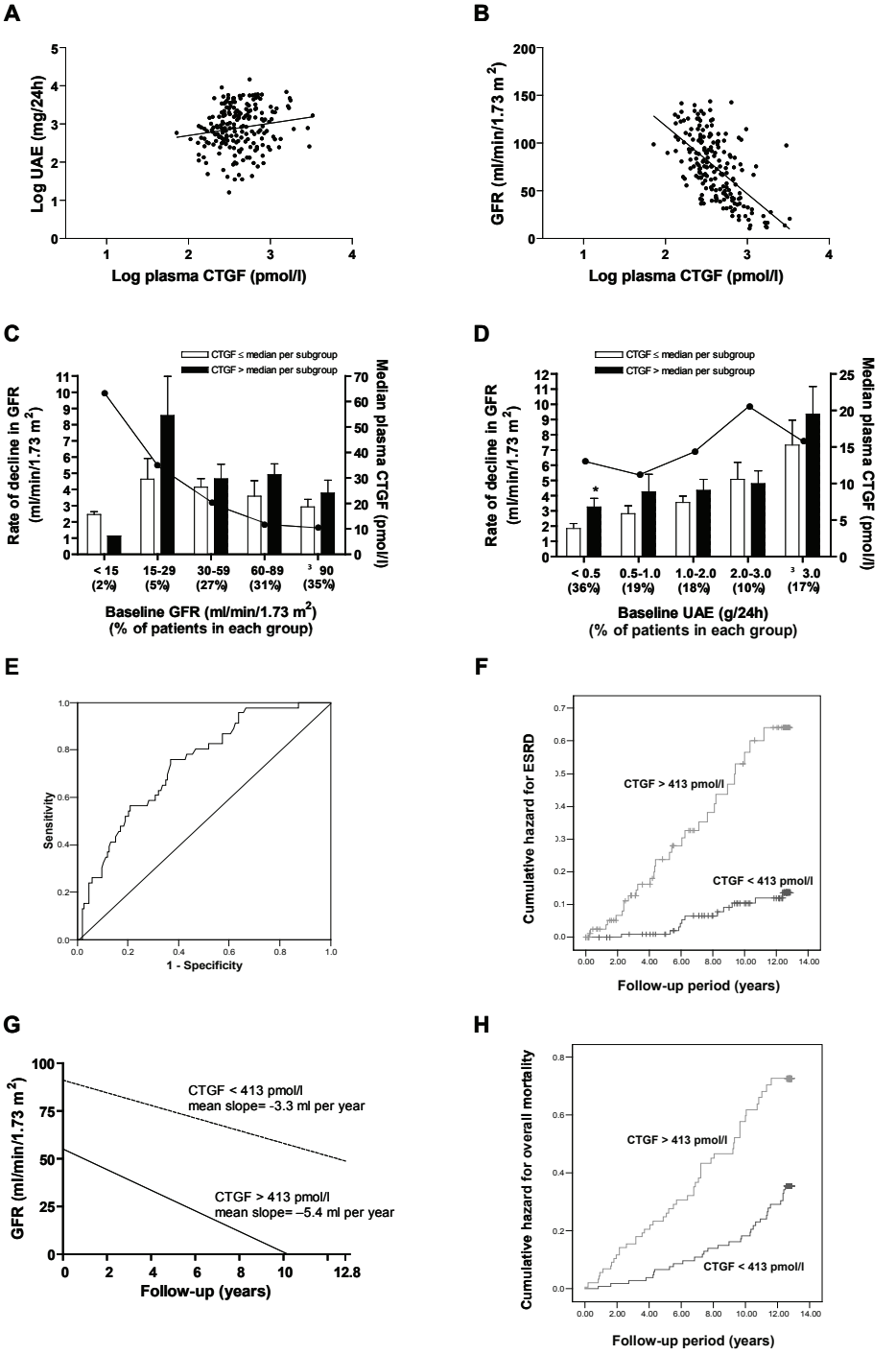
A
Log UAE (mg/24h)
Log plasma CTGF (pmol/l)
B
GFR (ml/min/1.73 m2)
Log plasma CTGF (pmol/l)
C
CTGF ≤ median per subgroup
CTGF > median per subgroup
Rate of decline in GFR (ml/min/1.73 m2)
Median plasma CTGF (pmol/l)
< 15 (2%)
15-29 (5%)
30-59 (27%)
60-89 (31%)
³ 90 (35%)
Baseline GFR (ml/min/1.73 m2) (% of patients in each group)
D
CTGF ≤ median per subgroup
CTGF > median per subgroup
Rate of decline in GFR (ml/min/1.73 m2)
Median plasma CTGF (pmol/l)
< 0.5 (36%)
0.5-1.0 (19%)
1.0-2.0 (18%)
2.0-3.0 (10%)
³ 3.0 (17%)
Baseline UAE (g/24h) (% of patients in each group)
E
Sensitivity
1 - Specificity
F
Cumulative hazard for ESRD
CTGF > 413 pmol/l
CTGF < 413 pmol/l
Follow-up period (years)
G
GFR (ml/min/1.73 m2)
CTGF < 413 pmol/l
mean slope= -3.3 ml per year
CTGF > 413 pmol/l
mean slope= –5.4 ml per year
Follow-up (years)
H
Cumulative hazard for overall mortality
CTGF > 413 pmol/l
CTGF < 413 pmol/l
Follow-up period (years)

Table 3. Cox proportional hazard model of baseline risk factors for overall mortality in patients with diabetic nephropathy

	Hazard ratio (95% CI)	P value
Sex (male vs. female)	2.03 (1.18–3.48)	0.011
HbA1c (per 1-SD increase = 1.5%)	1.47 (1.16–1.86)	0.001
Systolic blood pressure (per 1-SD increase = 22 mm Hg)	1.41 (1.10–1.80)	0.007
Plasma CTGF (per 1-SD increase = 1.9-fold)	1.39 (1.11–1.74)	0.005
Age (per 1-SD increase = 9.5 years)	1.37 (1.10–1.69)	0.004

Adjusted for smoking, history of major cardiovascular event, UAE, GFR, and all variables of this table.

vascular mortality. Although plasma CTGF at baseline was higher in normoalbuminuric patients who died during follow-up compared with those who were still in study (median 274 [interquartile range 202–392] vs. 230 [164–343] pmol/l, P=0.034), plasma CTGF was an independent predictor of neither overall mortality nor cardiovascular mortality in patients with normoalbuminuria.

Conclusions

The major findings in this study are that plasma CTGF level correlates with rate of decline in GFR and that it is an independent predictor of both ESRD and mortality in patients with type 1 diabetic nephropathy.

Baseline plasma CTGF was higher in patients with diabetic nephropathy than in patients with normoalbuminuria. This result is in accordance with our previous observations in a smaller study, in which plasma CTGF levels were increased in 10 patients with diabetic nephropathy (18). Although the correlations between plasma CTGF and UAE (R=0.16) and between plasma CTGF and GFR (R=–0.58) are relatively weak, the association of diabetic nephropathy with plasma CTGF is of similar strength as its association with the

Figure 1. Baseline plasma CTGF in patients with diabetic nephropathy. A: Log plasma CTGF correlates with log UAE (R=0.16, P=0.02). B: Log plasma CTGF correlates inversely with GFR (R=−0.58, P<0.001). C: Analysis per K/DOQI subgroup for chronic kidney disease. Within each subgroup, patients with plasma CTGF above the median tend to have a higher rate of decline in GFR. D: Analysis per subgroup for UAE. Within each subgroup, patients with plasma CTGF above the median tend to have a higher rate of decline in GFR. *P<0.05 vs. group with plasma CTGF below the median. E: Area under the ROC curve for prediction of ESRD by plasma CTGF is 0.74. With a cutoff value for plasma CTGF of 413 pmol/l, ESRD is predicted with a sensitivity of 73% and a specificity of 63%. F: Kaplan-Meier curve for prediction of ESRD. Cumulative hazard for ESRD is higher in patients with plasma CTGF >413 pmol/l than in patients with plasma CTGF <413 pmol/l. Log-rank test, P<0.001. G: Graphic illustration of the relation between plasma CTGF and GFR at baseline, and rate of decline in GFR during 12.8 years of follow-up. The mean rate of decline in GFR is higher in patients with high plasma CTGF (>413 pmol/l, solid line, 5.4±4.9 ml/min per 1.73 m^2 per year) than in patients with low plasma CTGF (<413 pmol/l, dashed line, 3.3±3.5 ml/min per 1.73 m^2 per year). Regression lines were computed from all available data points. The x-intercept value of 10.2 years for the high plasma CTGF group indicates mean time to ESRD. H: Kaplan-Meier curve for prediction of overall mortality. Cumulative hazard for overall mortality is higher in patients with plasma CTGF >413 pmol/l than in patients with plasma CTGF <413 pmol/l. Log-rank test, P<0.001.

established risk factors HbA1c and systolic blood pressure. Of interest, the OR for diabetic nephropathy of elevated plasma CTGF is of magnitude comparable to that of increased urinary CTGF excretion observed in a previous study (OR=2.0 and 2.3, respectively) (20). However, because urine and plasma samples have not been available from the same patients thus far, the relation between CTGF levels in plasma and urine remains to be determined in future studies.

In normoalbuminuric patients, renal function remained well preserved, and progression to ESRD was not observed during the follow-up period. However, in patients with diabetic nephropathy, renal function deteriorated progressively, and 21% developed ESRD. Consistent with previous reports, rate of decline in patients with overt diabetic nephropathy was most strongly associated with UAE (R=0.43) (28;29). Of interest, addition of plasma CTGF increased this correlation to a cumulative R of 0.46, whereas no such increase was observed with addition of any other baseline parameter, including baseline GFR. Accordingly, patients with high plasma CTGF levels had a steeper slope of decline in GFR than those with low plasma CTGF (cutoff 413 pmol/l).
Baseline plasma CTGF was identified as an independent parameter, but its association with decline in renal function was much stronger in patients with severe proteinuria, than in those with mild proteinuria. Separate analysis of patients with nephrotic range albuminuria revealed that plasma CTGF was the only independent predictor of ESRD, whereas differences even in GFR or UAE no longer predicted outcome in this subgroup. On the other hand, despite overlapping plasma CTGF levels, no such correlation or predictive value was found in patients with normoalbuminuria. It thus appears that plasma CTGF has unique potential as a prognostic biomarker of renal function decline, especially in diabetic patients with severe proteinuria.

Previously, we have observed that urinary CTGF excretion is elevated only in macro-albuminuric and not in microalbuminuric and normoalbuminuric patients (20). Together with the well established profibrotic effects of CTGF on tubular epithelial cells (8;30), these observations suggest that progressive loss of renal function might relate to excess plasma CTGF leaking into the urine in patients with severe proteinuria. It would be interesting to study whether levels of CTGF in unselected normoalbuminuric or microalbuminuric patients could identify subjects at high risk for progression of albuminuria, but this could not be investigated in the present study as the normoalbuminuric control group was selected for having long diabetes duration and thus low risk of progression of albuminuria. A microalbuminuric group was not available for this study.

In summary, addition of plasma CTGF to conventional risk factor assessment significantly improves prediction of ESRD and mortality in patients with overt type 1 diabetic nephropathy. Its unique predictive value for disease progression in patients with diabetic nephropathy, in particular those with heavy proteinuria, suggests that plasma CTGF might have clinical application as a biomarker. In addition, our findings lend further support to the notion that CTGF is an important pathogenic factor in progression of human diabetic nephropathy, consistent with previous observations in preclinical models.

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Chapter IV

Urinary Excretion of CTGF Predicts Deterioration of Renal Function in Idiopathic Membranous Nephropathy

J Aalten[1], TQ Nguyen[2], FA van Nieuwenhoven[2], D Li[3], R Goldschmeding[2], and JFM Wetzels[1]

[1]Department of Nephrology, Radboud University Nijmegen Medical Center, The Netherlands
[2]Department of Pathology, University Medical Center Utrecht, The Netherlands
[3]FibroGen, Inc., South San Francisco, CA, USA

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Abstract

There is much evidence that connective tissue growth factor (CTGF) is an important profibrotic protein in renal disease. In the present study, we evaluated urinary CTGF excretion (uCTGF) and its relation to progression of renal disease in patients with idiopathic membranous nephropathy (iMN).

For this prospective study, we selected patients with nephrotic iMN and normal serum creatinine at presentation. Urinary CTGF levels were determined by an enzyme-linked immunosorbent assay. To evaluate outcome, we defined renal death as a rise of serum creatinine to >1.5 mg/dl or a persistent rise of serum creatinine of >50% within the observation period.

Fifty-one patients were included (35M, 16F), age 47±15 yr, serum creatinine 0.99±0.22 mg/dl, serum albumin 2.4±0.5 g/dl, and proteinuria 8.4 (2.0–18.6) g/24 h. Mean follow-up time was 55±23 months. Urinary CTGF levels were detectable in 43 of 51 patients and the median uCTGF was 0.61 (0.06–92) μg/g creatinine. Patients in the highest quartile of uCTGF were significantly older (59±14 vs. 43±13 years) and had a higher baseline serum creatinine (1.26±0.14 vs. 0.89±0.15 mg/dl) and proteinuria (14.3 (3.0–16.8) vs. 7.1 (2.0–18.6) g/24 h). An ROC-curve for uCTGF as a predictor of the endpoint renal death identified an optimum sensitivity and specificity of 78% and 93%, respectively, at a threshold of 0.82 μg/g creatinine (AUC=0.85). A very strong correlation was identified between uCTGF and urinary β-2-microglobulin ($R=0.90$, $P<0.001$).

Thus, in patients with iMN, uCTGF is an accurate predictor of renal outcome. Our data suggest that, in iMN, uCTGF mainly reflects altered tubular reabsorption.

Introduction

Connective tissue growth factor (CTGF; CCN-2) belongs to the CCN family of peptides. CTGF has profibrotic properties and acts mainly downstream of transforming growth factor (TGF)-β (1–3). In several studies, CTGF has been related to tubulointerstitial fibrosis and progression of renal failure. In animal and human renal biopsies, CTGF mRNA was upregulated at sites of glomerular inflammation and tubulointerstitial damage (4–6). Blocking CTGF expression by antisense oligodeoxynucleotides in rats with renal failure significantly attenuated the upregulation of fibronectin mRNA and the production of collagen I, and also decreased severity of diabetic nephropathy in mice (7;8).
In patients with type 1 diabetes mellitus and diabetic nephropathy, losartan decreased urinary CTGF excretion (uCTGF) (9), while in 5/6 nephrectomized rats, fluvastatin prevented upregulation of CTGF and extracellular matrix accumulation (10). Furthermore, in transversal studies of patients with diabetic nephropathy, uCTGF correlated with the severity of diabetic nephropathy (11–13). Taken together, these data have identified CTGF as a promising therapeutic target for the treatment of patients with renal disease (4;14).

In idiopathic membranous nephropathy (iMN), which is one of the most frequent primary glomerular diseases in adult patients, tubulointerstitial damage plays an important role in progression of the renal disease (15). In previous studies, we have shown that urinary β-2-microglobulin (uβ2m) excretion, as a marker for tubulointerstitial disease, was an independent predictor of renal outcome (16).
It is unknown whether uCTGF is increased in patients with iMN and if it is related to the severity and prognosis of the renal disease in these patients. In order to answer these questions, we determined uCTGF in a prospectively followed cohort of patients with iMN.

Results

Baseline characteristics
We studied 51 patients with biopsy proven iMN. Baseline characteristics are given in Table 1. In more than 90% of the patients, the baseline measurements were performed within 1 year after the renal biopsy. Patients were not treated with immunosuppressive medication before baseline measurements were done, except for one patient who had been treated with prednisone. Mean follow-up was 55±23 months.
In 43 patients (84.3%), uCTGF was above the detection limit. The median uCTGF level in these patients was 0.61 (0.06–92) μg/g creatinine. Thirty-nine patients (76%) were treated with an ACE-inhibitor or angiotensin-II-blocker. There was no significant difference in uCTGF levels between patients who were treated or not treated with an ACE-inhibitor or angiotensin II receptor blocker.
Patients with a uCTGF level in the highest quartile were significantly older, had a higher serum creatinine and serum albumin level, had more proteinuria, and a higher uβ2m at baseline compared to patients with uCTGF levels in the lower quartiles (Table 2).

Table 1. Baseline characteristics of patients with iMN (n=51)

Sex (M/F)	35/16
Age (year)	47 ± 15
ECC-24 h (ml/min)	90 ± 25
eGFR (ml/min per 1.73 m^2)	73 ± 22
Serum creatinine (mg/dl)	0.99 ± 0.22
Serum albumin (g/dl)	2.0 ± 0.6
Serum cholesterol (mg/dl)	325 ± 77
Proteinuria (g/24 h)	8.4 (2–18.6)
uCTGF (µg/g creatinine)	0.61 (0.06–91.89)
uβ2m (mg/g creatinine)	0.33 (0.08–54,90)
Urinary IgG (mg/g creatinine)	152 (15–2004)
Urinary albumin (mg/g creatinine)	4663 (375–1406)
ACE-inhibitor or ARB (%)	39 (76)

Data are means±SD or median (range). ECC, endogenous creatinine clearance; eGFR, estimated GFR (original MDRD formula).

Table 2. Baseline characteristics of patients with uCTGF in the highest quartile (≥2.16 µg/g creat) compared to patients in the lower quartiles (<2.16 µg/g creat)

Parameter	uCTGF (≥2.16 µg/g creat)	uCTGF (<2.16 µg/g creat)	P value
Sex (M/F)	12/1	24/15	<0.05
Age (yr)	59 ± 14	43 ± 13	<0.01
Serum creatinine (mg/dl)	1.26 ± 0.14	0.89 ± 0.15	<0.01
Serum albumin (g/dl)	1.5 ± 0.4	2.2 ± 0.5	<0.01
Proteinuria (g/24 h)	14.3 (3.0–16.8)	7.1 (2.0–18.6)	<0.01
uCTGF (µg/g creatinine)	13.1 (2.16–91.9)	0.52 (0.06–2.05)	<0.01
uβ2M (mg/g creatinine)	17.6 (1.1–54.9)	0.19 (0.08–2.54)	<0.01

Data are means±SD or median (range).

Baseline uCTGF is higher in patients who reach renal death

Twenty-two patients (43.1%) reached the predefined endpoint of renal death within the observation period. Median time to the occurrence of renal death was 6.2 (0.2–33.4) months. In 18 patients, renal death was established based on a serum creatinine above 1.5 mg/dl. In 4 other patients, renal death was established based on an increase of serum creatinine of more than 50%. In the patients who reached the endpoint renal death, mean increase in serum creatinine was 0.49±0.2 mg/dl. In the non-failure group, mean

increase in serum creatinine was 0.04±0.12 mg/dl. In the failure group, estimated GFR was 60.1±18.9 ml/min at baseline and 37.7±8.2 ml/min at the time of the endpoint renal death. In patients who reached the endpoint renal death, baseline uCTGF was significantly higher than in patients not reaching the endpoint (median uCTGF 2.9 (0.15–91.9) vs. 0.5 (0.06–2.16) µg/g creatinine respectively, P<0.05).
In 29 control subjects, median uCTGF was 1.4 µg/g creatinine (range 0.04–3.59). These levels were higher as compared to patients with iMN who did not reach the endpoint renal death (P<0.01).

Baseline uCTGF is an independent predictor of renal death
An ROC-curve for prediction by uCTGF of the endpoint renal death identified an optimum sensitivity and specificity of 78% and 93%, respectively, at a threshold of 0.82 µg/g creatinine (AUC=0.85) (Figure 1A). Using this threshold level, the negative and positive predictive value of uCTGF were 84% and 89%, respectively. Renal survival was significantly better in patients with uCTGF under the threshold level (P<0.001, Figure 1B).

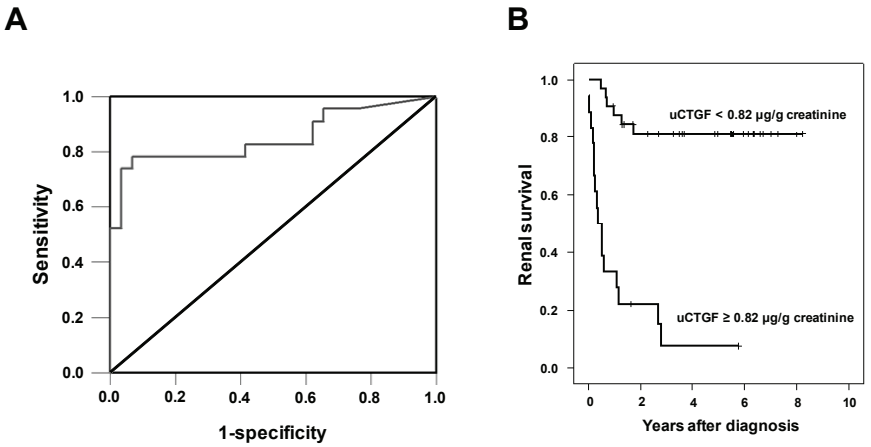

Figure 1. ROC and Kaplan-Meier curves for prediction of renal death in patients with iMN. A: ROC curve for prediction of renal death by uCTGF at a threshold of 0.82 µg/g creatinine identifies an optimum sensitivity and specificity of 78% and 93%, respectively. Area under the curve=0.85. B: Renal survival is higher in patients with uCTGF level above 0.82 µg/g creatinine than in patients with uCTGF below 0.82 µg/g creatinine. Log-rank test, P<0.001.

We performed multivariate Cox proportional hazard analysis to analyze which factors would independently predict renal outcome. Urinary albumin, uCTGF, and urinary IgG were log-transformed because they were not normally distributed. First, by univariate analysis, serum creatinine (P<0.001), proteinuria (g/24 h) (P<0.001), serum albumin (P<0.001), log uCTGF (P<0.001), log urinary IgG (P=0.001), log urinary albumin (P<0.001), and log uβ2m (P<0.001) were all found to be significantly related to renal

death. In the subsequent multivariate analysis, log uCTGF was identified as an independent predictor of renal death (hazard ratio 3.8 (95% CI 1.7–8.9), P=0.002). Other independent predictors for renal death were proteinuria (hazard ratio 1.13 (95% CI 1.004–1.26), P=0.043) and serum albumin (hazard ratio 0.14 (95% CI 0.031–0.65), P=0.012). Because uβ2m and uCTGF were highly correlated, they did not qualify as independent parameters. For this reason, uβ2m was not included in the multivariate analysis.

Urinary CTGF excretion correlates with uβ2m

As mentioned above, uCTGF was strongly correlated to uβ2m (R=0.90, P<0.001) (Figure 2A). Urinary CTGF excretion was also correlated to the urinary concentration of α1-microglobulin (R=0.83, P<0.001) and IgG (R=0.81, P<0.001), serum creatinine (R=0.50, P<0.001), GFR (R=0.42, P<0.01), and proteinuria (R=0.38, P<0.01).

Plasma samples were available from 18 patients. In these patients, we determined plasma CTGF levels (median 6.8 (1.8–31.3) ng/ml). No significant difference in plasma CTGF levels between patients who were treated with an ACE inhibitor or angiotensin-II-receptor antagonist and patients who were not treated with this medication were observed. Plasma levels in 21 healthy controls were significantly lower (3.7 (0.8–6.9) ng/ml, P<0.05). In contrast, plasma CTGF levels in non-progressors did not significantly differ from plasma levels in healthy controls. Plasma CTGF and uCTGF levels in iMN patients were significantly correlated (R=0.73, P<0.01). Although there was a correlation between plasma CTGF and plasma β2m (R=0.62, P<0.01), no significant correlation existed between plasma CTGF and renal function (plasma creatinine or estimated GFR). In these 18 patients, we calculated the fractional excretion of CTGF. The fractional excretion of CTGF was strongly correlated to the fractional excretion of β2m (R=0.99, P<0.001) (Figure 2B).

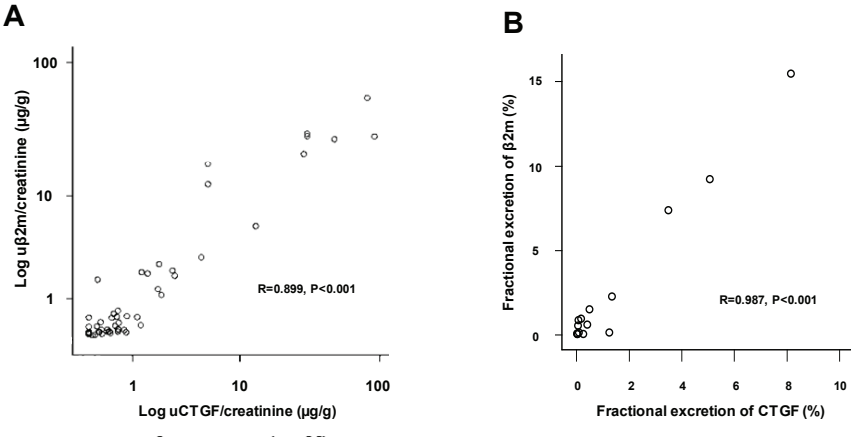

Figure 2. Correlation between uCTGF and uβ2m in patients with iMN. A: Urinary excretion of CTGF correlates with uβ2m (R=0.90, P<0.001). B: The fractional excretion of CTGF correlates with the fractional excretion of β2m (R=0.99, P<0.001).

Discussion

The main observations in this study are that CTGF is detectable in the urine of most patients with iMN and nephrotic range proteinuria, and that high uCTGF at baseline accurately predicts prognosis in terms of progression towards end-stage renal disease.

For optimal applicability of statistical analysis, a fixed threshold level of serum creatinine was used to identify renal death, as was done also in previous studies (16). Admittedly, this definition can be criticized since in patients with a relatively high baseline serum creatinine only slight increases would suffice to reach the end-point. To check whether we had correctly identified patients with progressive disease, we also calculated GFR from serum creatinine and urine collections. This confirmed that patients fulfilling the criterion of renal death had significant deterioration of renal function, with a mean estimated GFR decrease of 23 ml/min over the observation period.
In patients with iMN, deterioration of renal function is related to tubulointerstitial fibrosis (16). As mentioned previously, CTGF is a profibrotic protein and increased expression of CTGF has been observed in interstitial fibrotic areas from renal biopsies of patients with iMN (5). Based on these findings, high uCTGF in patients with progressive disease might be expected to derive from increased expression of CTGF in areas of tubulointerstitial fibrosis. However, our data do not support this notion. Urinary CTGF excretion was highly correlated with uβ2m and the fractional excretion of CTGF was correlated with the fractional excretion of β2m. In aggregate, these findings suggest that elevated uCTGF is a marker of more advanced tubulointerstitial disease, and mainly reflects decreased tubular reabsorption.

Interestingly, median uCTGF in patients with iMN who did not reach renal death was significantly lower as compared to median uCTGF in 29 healthy controls. Plasma CTGF levels were comparable to levels in controls and therefore cannot explain the lower uCTGF in non-progressors. Theoretically, lower uCTGF might derive also from increased reabsorption of CTGF in the (proximal) tubules, possibly triggered by proteinuria in patients with iMN. However, the high-normal values of uβ2m in non-progressive iMN patients argues against this option. Thus, it seems most likely that the lower uCTGF in non-progressive iMN patients reflects decreased renal expression of CTGF. Interestingly, also for TGF-β, a decreased urinary excretion was observed in patients with iMN as compared to healthy controls (17). Accordingly, glomerular and tubulointerstitial expression of CTGF and TGF-β mRNA in non-progressive iMN patients was not upregulated as compared to progressive iMN patients (5;18).

We identified uCTGF as an independent predictor of deterioration of renal disease. Although the predictive value of uCTGF was not better than that of known predictors, in particular uβ2m (16), our results are of interest. CTGF, in contrast to the more established low-molecular protein markers, is known to be involved in the pathogenesis of renal disease progression. Impaired reabsorption due to proximal tubular dysfunction leads to exposure of the distal nephron to filtered CTGF. This might induce profibrotic effects, as has been observed in proximal tubular cells (19;20).

Thus, in addition to being a biomarker, increase of uCTGF due to tubular dysfunction might represent a novel mechanism of CTGF involvement in progression of iMN, and possibly other diseases of the kidney.

Materials and Methods

Subjects

In our center, patients with iMN are evaluated using a standard protocol. In these patients, urine and blood samples are collected at baseline following a previously described protocol (16). Blood and urine samples are stored at –70˚C. In brief, the patients come to the ward after an overnight fast. On the morning of the measurements, patients are not allowed to take diuretics and 375 to 500 ml of tap water is given to enforce diuresis. The patients remain supine during 2 h except for voiding. Timed urine samples are collected, and in the middle of the collection period, a blood sample is drawn.

For this study, we studied patients with biopsy-proven iMN evaluated from 1995 onward. We only included patients with a baseline serum creatinine ≤1.5 mg/dl and proteinuria of ≥2.7 g/g creatinine and/or serum albumin ≤3.0 g/dl. Patients treated with immunosuppressive medication other than oral prednisone were excluded. In addition, urine samples from 29 healthy subjects were used as controls.

Laboratory measurements

CTGF levels in urine and plasma were determined with a sandwich enzyme-linked immunosorbent assay (ELISA), using monoclonal antibodies against distinct epitopes on the NH2-terminal part of human CTGF (FibroGen, Inc., South San Francisco, USA), as described previously (11). Briefly, microtiter plates were coated with capture antibody and blocked with BSA. Urine samples were diluted two-fold and plasma samples were diluted ten-fold in assay buffer, and a 50-µl diluted sample was added to each well together with 50-µl CTGF detection antibody conjugated with alkaline phosphatase. After addition of a substrate, absorbance was read at 405 nm. Purified recombinant human CTGF (FibroGen) was used for calibration. With the ELISA, both full-length and N-fragments of CTGF are detected. The detection limit of the assay was 0.08 ng/ml, and intra- and interassay variations were 6 and 20%, respectively. CTGF was measured in samples stored at –70˚C. To exclude degradation of CTGF during storage, we analyzed CTGF in parallel samples of 12 patients, which were stored with a protease inhibitor. No significant differences were observed in uCTGF levels between samples with or without a protease inhibitor.

Serum and urine parameters were determined using standard automated laboratory techniques. Urinary β2m was measured using a previously described ELISA technique (21).

Calculations

Glomerular filtration rate (GFR) was estimated using 24-hr creatinine clearance. In addition, we estimated GFR using the original MDRD formula (22). Fractional excretion was calculated according to the formula ([uCTGF × pCreat]/[pCTGF × uCreat]) × 100.

Statistical analysis

Comparisons between groups were performed by Mann-Whitney test. Receiver operating characteristics (ROC) curves were created to determine the area under the curve (AUC) and to calculate the sensitivity and specificity of uCTGF using the most discriminative threshold. The negative and positive predictive values for the occurrence of renal death for uCTGF were calculated. Kaplan-Meier statistics were used to calculate renal survival. The log-rank test was used to compare renal survival between patients with a high and low level of uCTGF. Renal death was defined as an increase of serum creatinine >50% or an increase of serum creatinine >1.5 mg/dl.

In a Cox proportional hazard analysis, we analyzed which factors were independent predictive factors for the end-point renal death. Six factors which were predictive for renal death in prior studies (uβ2m, serum creatinine, serum albumin, proteinuria, urinary excretion of IgG, and albumin) and uCTGF were included in the analysis (16). All significant predictors were included in the multivariate analysis. A backward stepwise selection method was used to determine independent predictors.

Linear regression was used to analyze correlations between uCTGF and other known risk factors for deterioration of renal disease in iMN. Linear regression was also used to analyze the correlation between the fractional excretion of CTGF and β2m.

All values are given as means±SD or median (range) as appropriate. All statistics were performed using SPSS software, version 12.0.1. $P<0.05$ was considered significant.

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Chapter V

CTGF Inhibits BMP-7 Signaling Activity in Diabetic Nephropathy

TQ Nguyen[1], P Roestenberg[1], FA van Nieuwenhoven[1], N Bovenschen[1], Z Li[2], L Xu[2], N Oliver[2], J Aten[3], JA Joles[4], C Vial[5], E Brandan[5], KM Lyons[6], and R Goldschmeding[1]

[1]Department of Pathology, University Medical Center Utrecht, The Netherlands
[2]FibroGen, Inc., South San Francisco, CA, USA
[3]Department of Pathology, Academic Medical Center Amsterdam, The Netherlands
[4]Department of Nephrology, University Medical Center Utrecht, The Netherlands
[5]Department of Cell and Molecular Biology, Catholic University of Chile, Santiago, Chile
[6]Department of Developmental Biology, University of California, Los Angeles, CA, USA

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Abstract

Diabetic nephropathy is characterized by upregulation of connective tissue growth factor (CTGF; CCN-2) and downregulation of bone morphogenetic protein (BMP)-7. Although CTGF was shown to inhibit BMP-4, possible cross-talk between BMP-7 and CTGF has not been studied. We hypothesized that in diabetic nephropathy, CTGF acts as an inhibitor of BMP-7 signaling activity.

As compared to diabetic wild-type $CTGF^{+/+}$ mice, diabetic $CTGF^{+/-}$ mice had approximately 50% lower CTGF mRNA and protein, less severe albuminuria, no thickening of the glomerular basement membrane (GBM), and preserved matrix metalloproteinase (MMP)-activity. Although renal BMP-7 mRNA was similar in diabetic $CTGF^{+/+}$ and $CTGF^{+/-}$ mice, phosphorylation of the BMP-signal transduction protein Smad1/5 and expression of the BMP-target gene Id1 were lower in diabetic $CTGF^{+/+}$ mice. Moreover, renal Id1 mRNA expression correlated with albuminuria (R=–0.86) and MMP-activity (R=0.76). In normoglycemic mice, a decrease of pSmad1/5 in renal cortex was observed after intraperitoneal injection of CTGF. Also in cultured renal glomerular and tubulointerstitial cells, co-stimulation of BMP-7 with CTGF diminished BMP-7 signaling activity, as evidenced by lower levels of pSmad1/5, Id1 mRNA, and BMP-responsive element (BRE)-luciferase activity. Co-immunoprecipitation, solid-phase binding assay, and surface plasmon resonance analysis showed that CTGF binds BMP-7 with high affinity (Kd ~ 14 nM).

In conclusion, overexpression of CTGF inhibits BMP-7 signal transduction in the diabetic kidney, and contributes to altered gene transcription, as well as to reduced MMP-activity, GBM-thickening and albuminuria, which are all hallmarks of diabetic nephropathy.

Introduction

Connective tissue growth factor (CTGF) is considered an important factor in the development of diabetic nephropathy. The renal expression of CTGF mRNA and protein is upregulated in human and experimental diabetic nephropathy (1–3). In patients with diabetic nephropathy, both plasma CTGF levels and urinary CTGF excretion are increased and correlate with clinical markers of renal disease (4–7). Recently, it was demonstrated that specific downregulation of CTGF by antisense oligonucleotide treatment attenuated albuminuria and mesangial matrix expansion in experimental type 1 and type 2 diabetic nephropathy (8). These observations suggest that CTGF is a critical determinant of structural and functional damage in diabetic nephropathy.

Several mechanisms have been proposed for a pathogenic role of CTGF in diabetic nephropathy. Studies with renal cells demonstrated that CTGF is involved in diabetes-associated changes such as extracellular matrix synthesis, cell migration, cellular hypertrophy, and epithelial-to-mesenchymal transition (2;9–11). CTGF might exert these effects in diabetes by modulating the activity of other growth factors. For example, CTGF is known to enhance profibrotic activity of transforming growth factor (TGF)-β1 and insulin-like growth factor (IGF)-1, which involves physical interaction of CTGF with these growth factors (12;13). In contrast, binding to CTGF potently antagonizes the signaling activity of bone morphogenetic protein (BMP)-4 in osteogenesis assays and in embryonic patterning (12). In the kidney, the importance of several BMPs including BMP-4 has been demonstrated mainly in developmental studies (14–16). However, thus far, only BMP-7 has also been studied for its contribution as an antifibrotic and proregenerative factor in response to injury of the adult kidney (17;18). Renal expression of BMP-7 is progressively decreased during the course of human and experimental diabetic nephropathy, and in podocytes cultured in high glucose medium (19–21), while restoration of BMP-7 availability has resulted in prevention or even reversal of functional and structural changes of diabetic nephropathy (22–24). Although it has been hypothesized that CTGF might inhibit BMP-7 (25;26), this has not been addressed experimentally.

Therefore, we set out to investigate the impact of CTGF on BMP-7 signal transduction and target gene expression in experimental diabetic nephropathy and in cultured renal cells.

Results

BMP-7 deficiency in diabetic nephropathy is accompanied by decrease of pSmad1/5 and BMP-target gene expression

Induction of diabetes in C57BL6/J mice by intraperitoneal injection of streptozotocin resulted in characteristic features of diabetic nephropathy, including persistent hyperglycemia, increased HbA1c levels, proteinuria, structural changes of the kidney, and increased CTGF levels in kidney, urine, and plasma (3). In diabetic mice, renal cortical expression of BMP-7 mRNA was decreased 2.6-fold. This was accompanied by reduced levels of pSmad1/5 protein, and also BMP-7 downstream target Id1 was decreased (Figure 1).

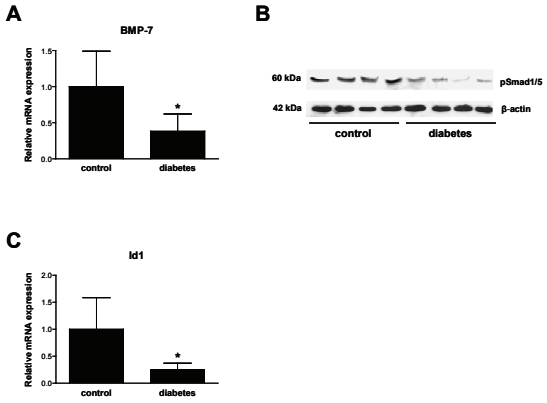

Figure 1. Decrease of BMP-7 signal transduction and target gene expression in diabetic nephropathy. Diabetes was induced in C57BL6/J mice by injection of streptozotocin. Renal cortex was harvested 9 wk after injection. Gene expression of BMP-7 and Id1 was evaluated by Q-PCR and pSmad1/5 protein level was analyzed by Western blotting. A-C: In diabetic mice, expression of BMP-7 was decreased 2.6-fold. This was accompanied by reduced levels of pSmad1/5 protein and Id1 mRNA. Data are mean±SD. *P<0.05.

CTGF$^{+/-}$ mice have lower levels of CTGF mRNA and protein

After 17 weeks of diabetes, CTGF mRNA and protein expression in renal cortex, as well as CTGF levels in plasma and urine, were increased in diabetic CTGF$^{+/+}$ mice as compared to non-diabetic CTGF$^{+/+}$ mice. In diabetic CTGF$^{+/-}$ mice, expression of CTGF mRNA and protein in renal cortex, and CTGF levels in plasma were not significantly different from non-diabetic mice. Although urinary CTGF excretion in diabetic CTGF$^{+/-}$ mice appeared to be lower than in diabetic CTGF$^{+/+}$ mice, this difference did not reach statistical significance. In non-diabetic CTGF$^{+/-}$ mice, CTGF expression seemed slightly lower than in non-diabetic CTGF$^{+/+}$, but this difference was not significant (Figure 2).
CTGF immunohistochemistry showed more prominent glomerular CTGF staining in diabetic CTGF$^{+/+}$ mice than in diabetic CTGF$^{+/-}$ mice and in non-diabetic mice. No prominent CTGF staining was observed in tubuli (Figure 3).

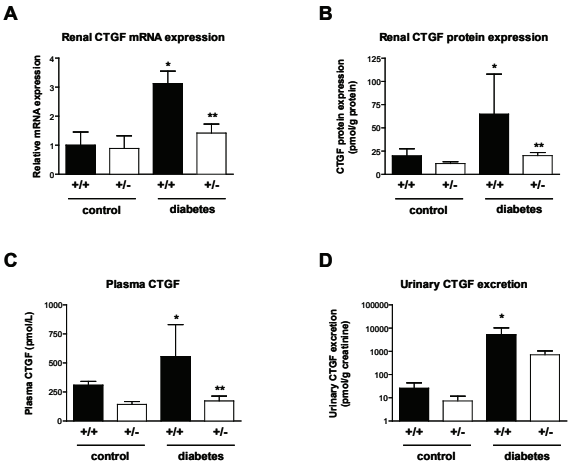

Figure 2. Levels of CTGF mRNA and protein are decreased in CTGF$^{+/-}$ mice. Diabetes was induced in CTGF$^{+/+}$ and CTGF$^{+/-}$ mice. A-D: CTGF mRNA and protein in renal cortex and CTGF levels in plasma were two-fold increased in diabetic CTGF$^{+/+}$ mice, as compared to diabetic CTGF$^{+/-}$ mice. Also urinary CTGF excretion tended to be lower in diabetic CTGF$^{+/-}$ mice than in diabetic CTGF$^{+/+}$ mice (P=0.29).

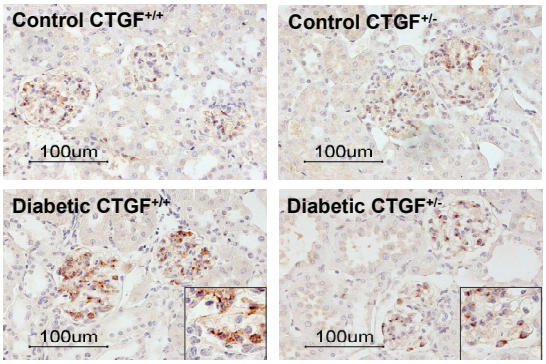

Figure 3. Renal CTGF protein expression is decreased in CTGF$^{+/-}$ mice. Diabetes was induced in CTGF$^{+/+}$ and CTGF$^{+/-}$ mice. CTGF immunohistochemistry showed more prominent glomerular CTGF staining in diabetic CTGF$^{+/+}$ mice than in diabetic CTGF$^{+/-}$ mice and non-diabetic mice.

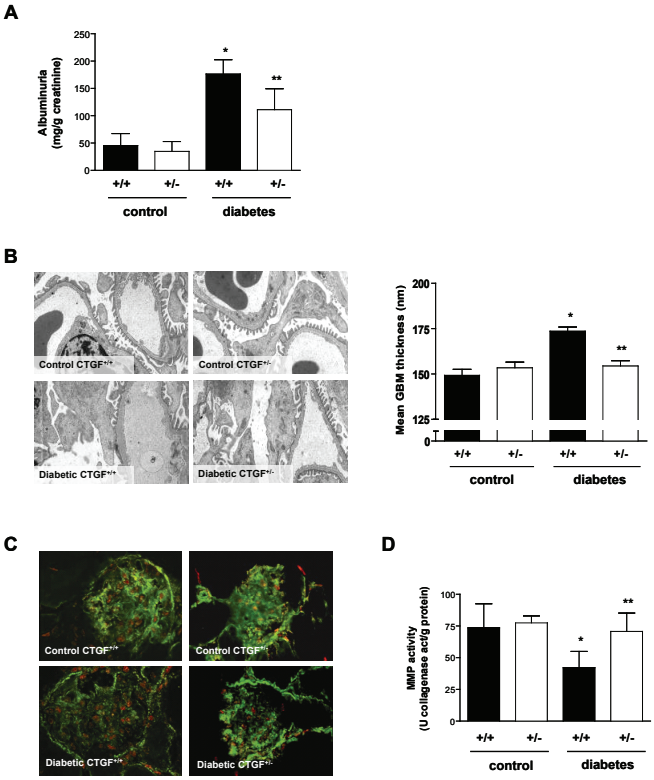

Figure 4. Diabetic nephropathy is attenuated in CTGF$^{+/-}$ mice. Diabetic nephropathy in CTGF$^{+/+}$ and CTGF$^{+/-}$ mice was assessed by albuminuria, GBM thickening, and MMP-activity. A: In diabetic CTGF$^{+/-}$ mice, albuminuria was significantly less pronounced than in diabetic CTGF$^{+/+}$ mice. B: GBM thickening in diabetic CTGF$^{+/+}$ mice, as determined by electron microscopy, was absent in diabetic CTGF$^{+/-}$ mice. *In situ* zymography on renal sections (green = MMP-activity, red = nuclear counterstain) (C) and colorimetric detection in renal lysates (D) showed that MMP-activity was decreased in diabetic CTGF$^{+/+}$ mice. In contrast, MMP-activity was preserved in diabetic CTGF$^{+/-}$ mice. Data are mean±SD. *P<0.05 versus control CTGF$^{+/+}$ mice, **P<0.05 versus diabetic CTGF$^{+/+}$ mice.

Diabetic nephropathy is attenuated in CTGF$^{+/-}$ mice

Albuminuria was increased in diabetic CTGF$^{+/+}$ mice as compared to non-diabetic mice. In diabetic CTGF$^{+/-}$ mice, albuminuria was significantly less pronounced than in diabetic CTGF$^{+/+}$ mice (111±39 vs. 176±26 mg/g creatinine, P=0.024; Figure 4A).

Examination of ultra-thin sections by electron microscopy showed that the thickness of the glomerular basement membrane (GBM) in diabetic CTGF$^{+/+}$ mice was increased compared to control CTGF$^{+/+}$ mice. However, no increase in GBM thickness was observed in diabetic CTGF$^{+/-}$ mice as compared to diabetic CTGF$^{+/+}$ mice (154±3.0 vs. 174±2.3 nm, P<0.01; Figure 4B).

In situ zymography showed that gelatinase activity, representing activity of matrix metalloproteinase (MMP)-2 and -9, was localized mainly in glomeruli. Activity of gelatinase was decreased in diabetic CTGF$^{+/+}$ mice, but was preserved in diabetic CTGF$^{+/-}$ mice (Figure 4C). This was confirmed by quantification of gelatinolytic activity in renal lysates, showing a reduction by approximately 40% in diabetic CTGF$^{+/+}$ mice (P=0.034), but not in diabetic CTGF$^{+/-}$ mice (Figure 4D).

Preserved BMP-signaling activity in diabetic CTGF$^{+/-}$ mice: correlation of CTGF level with albuminuria and MMP-activity

Renal cortical expression of BMP-7 mRNA was similar in diabetic CTGF$^{+/+}$ and CTGF$^{+/-}$ mice. Diabetic CTGF$^{+/-}$ mice had relatively preserved pSmad1/5 levels and Id1 mRNA expression as compared to diabetic CTGF$^{+/+}$ mice, whereas total Smad5 protein was not different (Figure 5A-C). Furthermore, Id1 mRNA correlated with albuminuria (R=−0.86, P=0.011) and with MMP-activity in renal lysates (R=0.76, P=0.037) (Figure 5D-E).

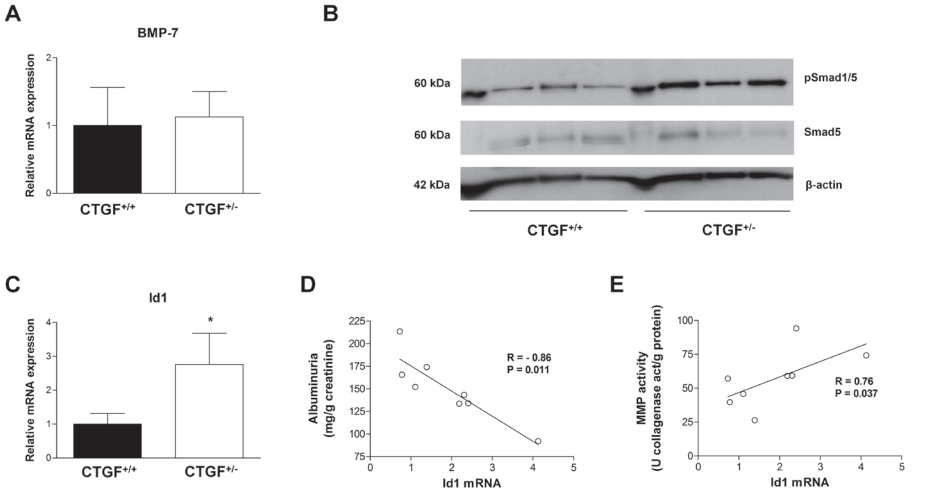

Figure 5. CTGF inhibits BMP-signaling activity in diabetic nephropathy. Renal cortex of diabetic CTGF$^{+/+}$ and CTGF$^{+/-}$ mice was harvested 17 weeks after induction of diabetes. A: Renal cortical expression of BMP-7 mRNA was similar in diabetic CTGF$^{+/+}$ and CTGF$^{+/-}$ mice. B and C: In diabetic CTGF$^{+/-}$ mice, pSmad1/5 protein and Id1 mRNA were higher than in diabetic CTGF$^{+/+}$ mice. D and E: Id1 mRNA correlated with albuminuria and MMP-activity. Data are mean±SD. *P<0.05.

Injection of recombinant CTGF impairs renal cortical BMP-7 activity in non-diabetic mice

Intraperitoneal injection of recombinant CTGF in non-diabetic mice resulted in transient elevation of plasma CTGF levels (Figure 6A). Although renal cortical expression of BMP-7 and Id1 mRNA was not different at the various time points (data not shown), pSmad1/5 protein levels were significantly decreased 4 hrs after injection (P=0.0083; Figure 6B). Injection with vehicle did not alter renal pSmad1/5 (Figure 6C).

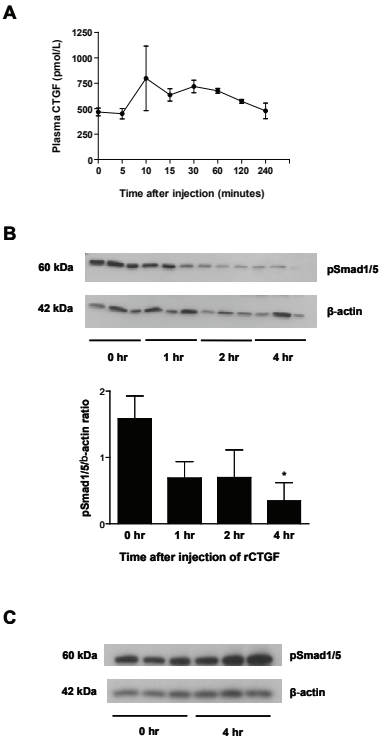

Figure 6. Injection of CTGF impairs renal cortical BMP-7 activity in non-diabetic mice. Recombinant CTGF, or vehicle only, was injected intraperitoneally in non-diabetic BALBc mice. A: CTGF levels in plasma were increased at 10 minutes after injection with rhCTGF. B: Renal cortical pSmad1/5 protein levels were significantly decreased 4 hrs after injection with rhCTGF. C: Renal cortical pSmad1/5 levels were not different 4 hrs after vehicle injection. Data are mean±SD. *P<0.05.

CTGF inhibits BMP-7 signal transduction and target gene expression in renal cells

Treatment of rat mesangial cells and HK-2 cells with BMP-7 resulted in increased phosphorylation of pSmad1/5 protein and Id1 mRNA expression. Addition of CTGF partially inhibited phosphorylation of Smad1/5 and reduced expression of Id1 mRNA (Figure 7A-D). Also in mouse podocytes, BMP-7 stimulation resulted in significant, although less pronounced, increase of Id1 mRNA expression, which was reduced by addition of CTGF (Figure 7E).

CTGF also inhibited activation of a BMP-responsive element (BRE)-luciferase construct, which specifically reports Smad1/5 mediated gene transcription (27). When TK173 cells were co-transfected with BRE-luciferase and pCAGGS-mCTGF, BMP-7 induced luciferase activity was significantly lower than in cells co-transfected with pCAGGS-lacZ control vector (Figure 7F).

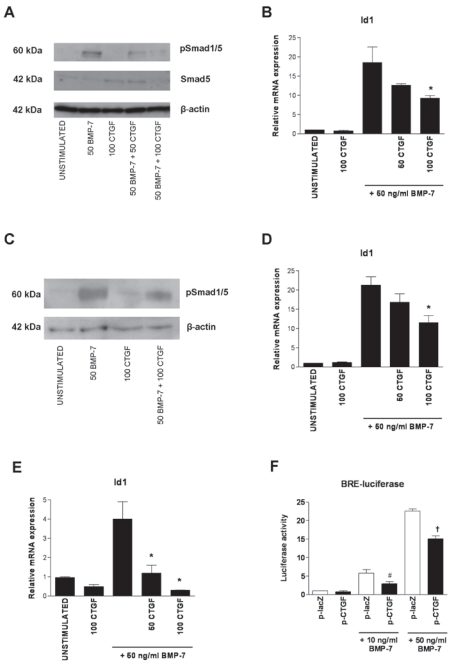

Figure 7. CTGF inhibits BMP-7 signal transduction and target gene expression in renal cells. Effects of CTGF on BMP-7 were studied in rat mesangial cells, mouse podocytes, proximal tubular epithelial cells (HK-2), and renal interstitial fibroblasts (TK173). A and B: Treatment of rat mesangial cells with BMP-7 resulted in increase of Smad1/5 phosphorylation and Id1 mRNA expression, which were both inhibited by co-stimulation with CTGF. C and D: Treatment of HK-2 cells with BMP-7 resulted in increase of pSmad1/5 protein and Id1 mRNA, which were both inhibited by co-stimulation with CTGF. E: Treatment of podocytes with BMP-7 resulted in increase of Id1 mRNA, which was inhibited by co-stimulation with CTGF. F: TK173 cells were transfected with the BMP-responsive element (BRE)-luciferase construct. Treatment with BMP-7 of cells co-transfected with control vector pCAGGS-lacZ resulted in increase of BRE-luciferase activity, whereas co-transfection with pCAGGS-CTGF inhibited BMP-7-induced luciferase activity. Data are mean±SD. *P < 0.05 vs. only 50 ng/ml BMP-7. #P < 0.05 vs. p-lacZ with 10 ng/ml rhBMP-7. †P < 0.05 vs. p-lacZ with 50 ng/ml BMP-7.

CTGF binds BMP-7 with high affinity

Co-immunoprecipitation experiments showed that captured BMP-7 on anti-BMP-7 mAb-coated agarose beads was able to bind CTGF. In the absence of BMP-7 only a weak CTGF band was detected, which is due to known aspecific binding of CTGF to agarose beads. Incubation with captured HGF on anti-HGF mAb-coated beads, and with BMP-7 and IgG1-coated beads, also did not result in significant pull-down of CTGF (Figure 8A). Similarly, solid-phase binding assay showed direct physical interaction between BMP-7 and CTGF, but not between HGF and CTGF, or bovine serum albumin (BSA) and CTGF. Increased binding between was observed as the concentration of either BMP-7 or CTGF was increased (Figure 8B).

The binding affinity of CTGF for BMP-7 was determined by surface plasmon resonance (SPR) analysis. CTGF displayed time-dependent association with immobilized BMP-7

followed by dissociation, which was dose-dependent as higher response was observed at higher CTGF concentrations (Figure 8C) and at higher BMP-7 density (data not shown). The data showed complex binding behavior in which multiple components were involved. Therefore, a heterogeneous two-site binding model was required to accurately describe the binding behavior. This resulted in Kd values describing a high and lower affinity component of 14 (±6) nM and 316 (±190) nM for the interaction between CTGF and BMP-7 [k_{on1}=2.4 (±0.9)×10^5 $M^{-1}s^{-1}$, k_{off1}=3.3 (±0.8)×10^{-3}, k_{on2}=1.3 (±0.5)×10^5, k_{off2}=4.0 (±1.8)×10^{-2}].

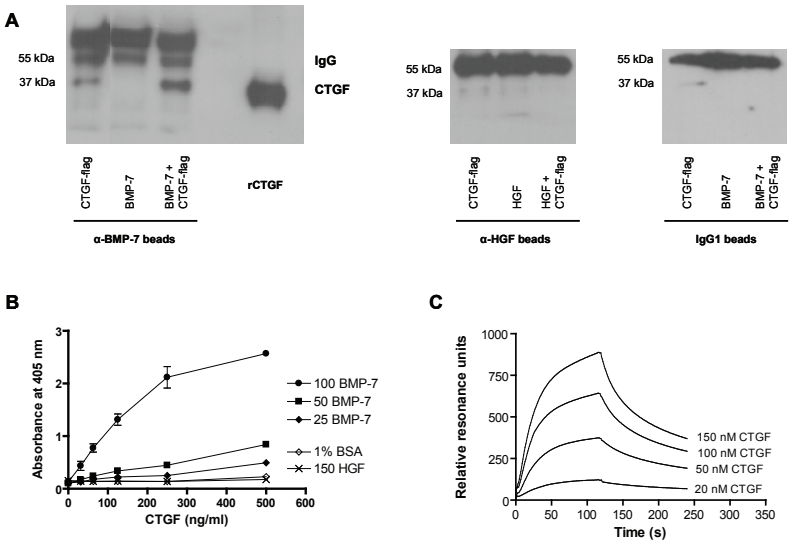

Figure 8. CTGF binds directly to BMP-7. Physical interaction between CTGF and BMP-7 was demonstrated by co-immunoprecipitation, solid-phase binding assay, and SPR. A: Anti-BMP-7 mAb-coated agarose beads were incubated with rhBMP-7 and/or rxCTGF-flag. As controls, beads were coated with anti-HGF and incubated with rhHGF, or coated with IgG1 and incubated with rhBMP-7, and/or rxCTGF-flag. Bound proteins were separated by SDS-PAGE and probed with anti-flag. Recombinant CTGF was run as control. B: Increasing concentrations of rhCTGF were added to microtiter plates coated with rhBMP-7, rhHGF, or 1% BSA. Bound proteins were detected with an AP-conjugated antibody against rhCTGF. C: Purified CTGF protein was run over BMP-7 sensor chips. Association and dissociation were monitored by a change in the resonance units.

Discussion

The results of this study reveal how diabetes-induced increase of CTGF expression contributes to impairment of renal BMP signaling activity, and that this is associated with severity of structural and functional hallmarks of diabetic nephropathy.

To determine how CTGF expression level might relate to reduced BMP-7 signaling in diabetic nephropathy, we compared pSmad1/5 and Id1 levels in diabetic CTGF$^{+/+}$ mice

with those in diabetic CTGF$^{+/-}$ mice. The latter mice have lower CTGF expression in kidney, plasma, and urine as compared to diabetic CTGF$^{+/+}$ mice, but they have equal TGF-β1 expression (data not shown). Thus, diabetic CTGF$^{+/-}$ mice constitute a unique model to assess the impact of CTGF level on downstream BMP-7 activity in diabetic nephropathy. BMP-7 mRNA expression in renal cortex was similar in diabetic CTGF$^{+/+}$ and CTGF$^{+/-}$ mice, indicating that CTGF level does not influence BMP-7 expression. However, pSmad1/5 protein and Id1 mRNA levels were lower in diabetic CTGF$^{+/+}$ mice, as compared to diabetic CTGF$^{+/-}$ mice. This indicates that CTGF might be an important determinant of the diabetes-induced reduction in signaling activity of residual BMP-7. CTGF is known to also directly modulate BMP-4 and TGF-β1 (12). In addition, the level of BMP signaling activity in the kidney is subject to the influence of other members of the TGF-β superfamily, BMP receptors, and of BMP modulators like gremlin, noggin, kielin/chordin-like protein, or uterine sensitization-associated gene-1 (28). Therefore, the relative contribution of BMP-7 inhibition by CTGF remains to be established.

Albuminuria, thickening of the GBM, and decreased activity of MMPs, are all hallmarks of human and experimental diabetic nephropathy (29–31). Recently, it was shown that renal CTGF protein correlates with GBM thickness and prospective albuminuria in a non-human primate model of diabetes (32). We observed that these alterations, and also decreased MMP activity, were attenuated or absent in diabetic CTGF$^{+/-}$ mice. This indicates that CTGF plays a pathogenic role in at least these characteristic manifestations of diabetic nephropathy. Similarly, decrease of albuminuria in experimental models of type 1 and type 2 diabetic nephropathy has been observed in mice treated with an anti-CTGF antibody, and with a CTGF antisense oligonucleotide, respectively (8;33). In the latter study, inhibition of CTGF also resulted in reduction of serum creatinine and inhibition of mesangial matrix expansion, which were not observed in our diabetic CTGF$^{+/-}$ mouse model (data not shown). A possible explanation for this discrepancy is that CTGF level derived from a single functional allele might suffice to mediate diabetes-induced increase in serum creatinine and matrix accumulation. Treatment with antisense oligonucleotides might have lowered CTGF availability to levels below those in our diabetic CTGF$^{+/-}$ mice resulting in more complete protection. However, we cannot directly compare actual reduction of CTGF levels in the different studies, and BMP-signaling activity was not addressed after anti-CTGF antibody or CTGF-antisense oligonucleotide treatment.

The strong correlation of Id1 with both albuminuria and MMP-activity suggests that CTGF-dependent suppression of this target gene of BMP signaling might be involved directly in the pathogenesis of diabetic nephropathy. Accordingly, diabetic transgenic mice overexpressing BMP-7 in podocytes and proximal tubular epithelial cells had higher Id1:PAI mRNA ratio and preserved MMP-activity, and developed less albuminuria, as compared to diabetic wild-type mice (23).
As in overexpression of endogenous CTGF in diabetic mice, injection of recombinant human CTGF in non-diabetic mice also resulted in decrease of pSmad1/5. However, this did not affect levels of Id1 mRNA. Thus, transient two-fold increase of plasma CTGF in a non-diabetic environment appeared not to be sufficient for inhibition of Id1 mRNA, which might require higher or more sustained elevation of CTGF, or additional diabetes-

induced changes.
As for the nature of CTGF-BMP-7 interaction in the kidney, exogenous CTGF protein as well as CTGF transfection inhibited BMP-7 signaling activity in cultured mesangial cells, podocytes, proximal tubular epithelial cells, and also in renal interstitial fibroblasts. This was exemplified by inhibition of BMP-7-induced pSmad1/5, Id1, and BRE-luciferase activity. The inhibitory effect of CTGF was robust, in the sense that it was observed with exogenously added human and *Xenopus* CTGF, as well as with transfected mouse CTGF.
Direct physical interaction between CTGF and BMP-7 was evidenced by co-immunoprecipitation and in a solid-phase binding assay. Furthermore, SPR analysis demonstrated that the interaction between CTGF and BMP-7 was complex and consisted of a high and a lower affinity component (Kd values of 14 nM and 316 nM, respectively). This may not only result from inherent biological properties of both CTGF and BMP-7, but may also be due to partial blocking of CTGF interactive sites during the immobilization of BMP-7. The high binding affinity of CTGF and BMP-7 was comparable to that described for CTGF and BMP-4, which had a Kd value of 5 nM (12).

In conclusion, overexpression of CTGF inhibits BMP-7 signal transduction in the diabetic kidney, and contributes significantly to altered gene transcription, as well as to reduced MMP-activity, and to GBM thickening and albuminuria, which are all hallmarks of diabetic nephropathy.

Concise Methods

Animal experiments
Signaling activity of BMP-7 was studied in diabetic mice (3). Briefly, diabetes was induced in nine 12-wk old female C57Bl/6J mice by a single intraperitoneal injection of 200 mg/kg streptozotocin (Sigma, St. Louis, MO, USA) in sodium citrate buffer. Six control animals were injected with vehicle only. Hyperglycemia was determined 3 days after injection by measurement of blood glucose levels. Slow release insulin pellets (Linshin, Scarborough, Canada) were implanted to stabilize the condition of the diabetic animals. Mice were killed 9 weeks after injection. Renal cortex was harvested by dissecting small caps of the upper and the lower poles. Before homogenization, absence of medulla was checked in frozen sections of the cut surface.
Effects of CTGF level on BMP-7 signaling activity were studied in diabetic $CTGF^{+/+}$ and $CTGF^{+/-}$ mice. Outbred male BALBc/129Sv $CTGF^{+/-}$ mice, in which exon 1 of one CTGF allele has been replaced by a neomycin resistance gene (34), were mated with female C57Bl/6J mice. From their first offspring, female $CTGF^{+/-}$ mice and female $CTGF^{+/+}$ littermates were used for the present study. Diabetes was induced in five 16-wk old $CTGF^{+/-}$ mice and four $CTGF^{+/+}$ mice by injection of streptozotocin. Nine control mice were injected with vehicle only. After 9 weeks, unilateral nephrectomy was performed in all animals to aggravate the diabetic nephropathy model. Mice were killed 17 weeks after induction of diabetes. Albumin levels were determined by sandwich ELISA using a goat-anti-mouse albumin antibody (Bethyl Laboratories, Inc., Montgomery, TX, USA).

Urinary creatinine excretion was determined by enzymatic assays (J2L Elitech, Labarthe Inard, France).
Effects of CTGF on BMP-7 signaling activity in renal cortex of normoglycemic mice were studied in 24 BALBc mice, which were injected intraperitoneally with rhCTGF (FibroGen, Inc, South San Francisco, USA) at a dose of 20 μg/kg diluted in 50 mM Tris-HCl buffer containing 800 mM NaCl. Mice were killed 0, 5, 10, 15, 30, 60, 120, and 240 minutes after injection (3 mice for each time point). Six control mice were injected with vehicle only, and killed 0 and 240 minutes after injection.
All animals were housed in standard cages in a room with constant temperature, on a 12-h light-dark cycle. Animals were fed a standard pellet laboratory chow and had free access to water. The experiments were performed with the approval of the Experimental Animal Ethics Committee of the University of Utrecht.

CTGF ELISA
CTGF levels in plasma, urine, and renal lysates were determined by sandwich ELISA using 2 distinct specific antibodies (FibroGen) both directed against CTGF (3). The assay detects full-length and N-terminal fragments of CTGF. CTGF levels are expressed as pmol/l.

CTGF immunohistochemistry
CTGF immunohistochemistry was performed as described previously (3). Briefly, antigen retrieval was performed by predigestion with Protease XXIV (Sigma). Sections were incubated with a CTGF specific human monoclonal antibody (FibroGen), followed by incubation with rabbit-anti-human IgG (Dako, Glostrup, Denmark) and goat-anti-rabbit Powervision-PO (Klinipath, Duiven, The Netherlands). Bound antibody was visualized with Nova RED (Vector Laboratories, Burlingame, CA, USA).

Electron microscopy
Tissue samples were fixed in Karnovsky solution. Upon embedding, samples were rinsed with 0.1 M Na-cacodylatebuffer, followed by fixation with 1% osmiumtetroxide, and dehydrated with acetone and embedded in epon. Ultra-thin sections of 95 nm were cut and mounted on copper one-hole specimen support grids. Sections were stained with uranyl acetate and lead citrate to provide contrast.
Ultra-thin sections were photographed using a transmission electron microscope (JEM-1200 EX, JEOL, Peabody, MA, USA). GBM thickness was measured in 5 random glomeruli per mouse at 10 perpendicular cross-sections of GBM per glomerulus at a magnification of 5000x and analyzed by computer image analysis (ImageJ, NIH, www.rsb.info.nih.gov/ij/).

***In situ* zymography**
Glomerular MMP activity was visualized by *in situ* zymography and confocal laser-scanning microscopy. Frozen tissue sections were incubated with DQTM gelatin from pig skin (Invitrogen, Carlsbad, CA, USA), 1:20 diluted in 50 mM Tris-HCl buffer containing 10 mol/l CaCl2, 0.05% Brij 35 and 5 mmol/l PMSF, pH 7.4. Slides were incubated in a dark humidified chamber at 37ºC for 19 hrs. An MMP inhibitor, 1,10-phenanthroline

monohydrate (2 μg/ml), was used to verify that the obtained gelatinase activity specifically represented MMP activity. Nuclei were counterstained in red with propidium iodide.

Gelatinase activity in tissue lysates

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 (Invitrogen). Collagenase was used as standard and specificity of the gelatinase activity was verified using 1,10-phenanthroline monohydrate. Protein concentrations of the lysates were determined colorimetrically and used for normalization of collagenase activity per lysate.

Cell culture

The immortalized human proximal tubular epithelial cell line HK-2 (35), the SV40-transformed human renal fibroblast cell line TK173 (gift of F. Strutz, Göttingen, Germany) (36), rat mesangial cells (9), and conditionally immortalized mouse podocytes were used to study the effects of CTGF on BMP-7 signaling activity. HK-2, TK-173, and rat mesangial cells were maintained in DMEM with 10% fetal bovine serum (FBS), penicillin and streptomycin (Invitrogen). Podocyte cultures were maintained at 33°C in RPMI with 10% FBS in the presence of interferon-γ (IFN-γ) (R&D Systems, Abingdon, UK). For differentiation, podocytes were plated in collagen I coated wells at 37°C, and cultured for another 2 weeks in RPMI with 10% FBS in the absence of IFN-γ. HK-2, rat mesangial cells, and podocytes were seeded at a density of 2×10^5 cells per well in six-well plates. Cells were serum-starved for 24 h, followed by stimulation with and without 50 ng/ml rhBMP-7 (R&D Systems) in the presence of 0, 1, 50, or 100 ng/ml rhCTGF (FibroGen) or rxCTGF from *Xenopus laevis* (12). Cells were harvested after 1 h for Western blot analysis of pSmad1/5, and after 2 h for quantitative PCR.

For transfection experiments, TK173 cells were seeded at 1×10^5 cells per well in six-well plates. After overnight culture, cells were washed and transfected with the BRE-luciferase-reporter construct (kindly provided by P. ten Dijke, Leiden, The Netherlands). Cells were co-transfected with pCAGGS-CTGF or pCAGGS-lacZ. The plasmid pCAGGS-mCTGF was constructed by insertion of mouse CTGF cDNA into an EcoRI cloning site of the pCAGGS expression vector (kindly provided by J. Miyazaki, Osaka, Japan) (37). Transfection was carried out using Lipofectamine 2000 (Invitrogen) with 2.0 μg of reporter construct, 2.0 μg of pCAGGS plasmid, and 40 ng of pRL-TK-Renilla (Promega, Madison, WI, USA) as a control to normalize transfection efficiency. After 24 hours, cells were washed, serum-starved for 12 hours, and exposed to 10 or 50 ng/ml rhBMP-7 for 24 hours. Luciferase activity was quantified using the Dual-Luciferase Reporter 1000 Assay System (Promega).

Quantitative PCR

Total RNA was extracted from 30 mg frozen renal cortex, or from cultured cells, using RNeasy columns (Qiagen, Venlo, The Netherlands). After cDNA synthesis, expression of BMP-7, Id1, and CTGF mRNA, was assessed by quantitative real-time PCR using TaqMan Gene Expression Assays with predesigned probe and primers (Applied Biosystems, Foster City, CA, USA). TATA-box binding protein and β-actin were used as internal reference.

Western blot analysis
Cells or sections of renal cortex were homogenized in lysis buffer (20 mM Tris at pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 0.5% sodium deoxycholate, 50 mM NaF, 2mM Na3VO4) containing 5% Protease Inhibitor Cocktail (Sigma, St Louis, MO, USA). Protein quantity was determined by BCA protein assay kit (Pierce, Rockford, IL, USA). Samples were run on 8% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred onto PVDF membranes. Following blocking, membranes were incubated with polyclonal antibody specifically directed against pSmad1/5 or total Smad5 (Cell Signaling Technology, Beverly, MA, USA) overnight, washed, and incubated with horseradish peroxidase-conjugated secondary antibody. For detection, membranes were incubated with SuperSignal West Dura Chemiluminescent Substrate (Pierce). Actin antibody (Sigma) was used on the same blot for loading control. Phophorylated Smad1/5 and total Smad5 staining were performed on the same blots (with in between stripping) to control for possible regulation of total Smad5 expression.

Co-immunoprecipitation
Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were coated with monoclonal anti-BMP-7 (R&D Systems) and blocked with 1% BSA. Beads were subsequently pre-incubated with rhBMP-7 (R&D Systems) at 4°C. As controls, beads coated with monoclonal anti-HGF and pre-incubated with rhHGF (both R&D Systems), or beads coated with IgG1 and pre-incubated with rhBMP-7 were used. The next day, beads were washed and rxCTGF-flag was added for 4 h at 37°C. Beads were washed, resuspended in 20 µl of PBS, and bound proteins were eluted and denatured in SDS sample buffer and separated under reducing conditions by SDS-PAGE. Membranes were blocked and incubated with monoclonal anti-flag (Sigma). After incubation with horseradish peroxidase-conjugated rat-anti-mouse antibody, detection was performed as described above.

Solid-phase binding assay
Microtiter plates were coated overnight at 4°C with 0, 25, 50, and 100 ng/ml rhBMP-7, or with 150 ng/ml rhHGF. Wells were rinsed and blocked with 1% BSA for 2 h. After washing, a range of 0-500 ng/ml of rhCTGF (FibroGen) was added, followed by alkaline phosphatase-conjugated monoclonal antibody against human CTGF (FibroGen). After incubation for 2.5 h at 37°C, plates were washed and substrate solution containing p-nitrophenyl phosphate was added. Absorbance was read at 405 nm.

Surface plasmon resonance analysis
Real-time binding experiments were performed on the Biacore 2000 (GE Healthcare, Uppsala, Sweden). Carrier-free recombinant BMP-7 (R&D Systems) was immobilized on the CM5 sensor-chip surface at 66 and 122 fmol/mm^2. One control flow-channel was routinely activated and blocked in the absence of protein. Association of rhCTGF (5-300 nM) was assessed *in triplo* in 10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20 for 2 min, at a flow rate of 20 µl/min at 25°C. Dissociation was allowed for 2 min in the same buffer flow. Sensor chips were regenerated using several pulses of 20 mM HEPES (pH 7.4), 1 M NaCl at a flow rate of 20 µl/min. Data were

corrected for both refractive index changes and association and dissociation rate constants were determined by nonlinear regression analysis using the BIAevaluation Software 3.1 (GE Healthcare).

Statistical analysis

Data are presented as mean±SD. Differences between groups were analyzed by Student's *t*-test or ANOVA with Bonferroni correction for multiple comparisons. Correlations were assessed by linear regression. For all comparisons, a value of $P<0.05$ was considered to be significant (two-tailed).

Acknowledgments

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Chapter VI

Myofibroblast Progenitor Cells are Increased in Number in Patients with Type 1 Diabetes and Express Less BMP-6: A Novel Clue to Adverse Tissue Remodeling?

TQ Nguyen[1], H Chon[2], FA van Nieuwenhoven[1], B Braam[2], MC Verhaar[3], and R Goldschmeding[1]

[1]Department of Pathology, University Medical Center Utrecht, The Netherlands
[2]Department of Nephrology, University Medical Center Utrecht, The Netherlands
[3]Department of Vascular Medicine, University Medical Center Utrecht, The Netherlands

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Abstract

Aims/hypothesis: Growth factor imbalance and endothelial progenitor cell dysfunction are well-known elements of the inappropriate response to injury in human and experimental diabetes. We hypothesized that in diabetes the outgrowth of myofibroblast progenitor cells (MFPC) is also altered and that this relates to aberrant gene expression of growth factors involving members of the TGF-β/bone morphogenetic protein (BMP) superfamily.

Subjects and methods: MFPC were cultured from peripheral blood mononuclear cells of patients with type 1 diabetes and control subjects. Microarray analysis, quantitative PCR, and ELISA were used to identify differentially regulated TGF-β/BMP superfamily genes in diabetes- and control-derived MFPC. Possible effects of BMP-6 on TGF-β-induced gene expression were examined in cultured renal fibroblasts (TK173 cells).

Results: Blood from diabetic patients yielded higher numbers of MFPC than blood from control subjects (1.6-fold increase, $P<0.05$), involving increased proliferation and decreased apoptosis. BMP-6 mRNA and protein were downregulated in MFPC derived from patients with diabetes (3.9- and 1.8-fold decrease, respectively, $P<0.05$). Furthermore, an inverse correlation was observed between BMP-6 mRNA level and the number of MFPC in patients with diabetes ($R=-0.85$, $P<0.05$). In TK173 cells, BMP-6 antagonized the TGF-β-induced expression of the genes encoding plasminogen activator inhibitor-1 and connective tissue growth factor (70 and 50% reduction, respectively).

Conclusions/interpretation: Considering the importance of BMP-6 in processes such as angiogenesis and its novel anti-TGF-β effects, we propose that the excess numbers of BMP-6-deficient MFPC may favor adverse tissue remodeling in patients with diabetes, both numerically and by inappropriate orchestration of their microenvironment.

Introduction

Bone marrow-derived circulating progenitor cells (cPC) have not only been shown to be important contributors to the repair of damaged tissue (1;2) but are also involved in the pathological processes of tissue remodeling, such as atherosclerosis, fibrosis, and chronic transplant vasculopathy (3−6). In several of these conditions, myofibroblasts or smooth muscle cells driving adverse tissue remodeling originate largely from cPC (6). Recently it was demonstrated that, in addition to endothelial cells, smooth muscle cells can also be cultured from peripheral blood (7;8). Moreover, it was shown that the number of colony-forming units in endothelial progenitor cell (EPC) cultures correlated with cardiovascular function (9) and that the development of fibrocytes, defined as collagen-secreting cells present in peripheral blood, was upregulated in patients with burn wounds and that a positive correlation existed between fibrocyte numbers and serum TGF-β levels (10).

In diabetes the tissue response to injury is compromised, as evidenced by the development of excessive renal and cardiovascular damage and the ensuing matrix accumulation. Thus far, dysfunction of cPCs in patients with diabetes has been examined only at the EPC level, showing decreased outgrowth of diabetes-derived EPC and impaired angiogenic activity (11;12). Outgrowth of myofibroblast progenitor cells (MFPC) in patients with diabetes has not yet been investigated.

It seems likely that cPC dysfunction in diabetes relates, at least in part, to imbalance of growth factor signaling. In particular, several authors have demonstrated that TGF-β, a profibrotic cytokine and potent stimulator of myofibroblast differentiation, is elevated in patients with diabetes and that it functions as a crucial factor in the progression of diabetic nephropathy (13;14). Levels of plasminogen activator inhibitor-1 (PAI-1) and connective tissue growth factor (CTGF), both key mediators of profibrotic TGF-β activity, are also significantly increased in tissue and plasma of experimental and human diabetic nephropathy (15−20). Furthermore, Korpinen *et al.* have demonstrated increased secretion of TGF-β by 2-day cultured peripheral blood mononuclear cells (PBMC) from patients with type 1 diabetes and showed an association of these TGF-β levels with the occurrence of diabetic nephropathy (21). In contrast, renal expression of another member of the TGF-β superfamily, the antifibrotic bone morphogenetic protein (BMP)-7, is decreased during the course of experimental diabetic nephropathy (22), and *in vitro* studies with human mesangial cells have revealed that a high glucose concentration induces the expression of BMP antagonists CTGF and gremlin (23;24). The relevance of TGF-β/BMP signaling imbalance is underscored by a similar reduction in matrix accumulation and even the reversal of experimental diabetic nephropathy by the exogenous administration of BMP-7 or inhibition of TGF-β (25;26).

We hypothesized that, in diabetes, the TGF-β/BMP signaling imbalance is related to aberrant programming of cPC, leading to increased outgrowth and dysregulation of MFPC with consequent fibroplasia and scar formation instead of functional repair. Therefore, we determined the number of MFPC that could be cultured from peripheral blood of patients with diabetes and control subjects, and we set out to evaluate the possible role of TGF-β/

BMP superfamily members in functional and numerical differences between diabetes- and control-derived MFPC.

Subjects and Methods

Subjects

Six patients with type 1 diabetes, who visited the outpatient department of Vascular Medicine of the University Medical Centre in Utrecht, were entered into the study. The patients, three males and three females with a mean age of 37.4±7.0 years (ranging from 32 to 48 years), were all free of medication except for insulin therapy. The mean duration of diabetes was 21.7±10.8 years. Patient blood glucose levels were relatively well regulated (mean HbA1c of 7.1±0.3%) and patients did not show any signs of organ complications due to diabetes. As controls, we selected six healthy age- and sex-matched subjects (mean age 37.6±7.2 years).
All subjects gave informed consent. The study was performed according to the principles of the Declaration of Helsinki.

Culture of myofibroblast progenitor cells

MFPC were isolated and cultured according to protocols used by others for the culture of fibrocytes, i.e. collagen-secreting cells cultured from peripheral blood (10;27). Briefly, 20–40 ml peripheral blood samples of patients and control subjects were collected in tubes containing EDTA. Total PBMC were isolated by density gradient centrifugation on HistoPaque 1077 (Sigma, St Louis, MO, USA). PBMC were seeded on six-well plates coated with 0.5 μg/ml human fibronectin (Sigma) in PBS at a density of 5×10^6 cells per well and cultured in low-glucose (1 g/l) DMEM supplemented with 20% fetal bovine serum, l-glutamine (2 mmol/l), penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA) in a humidified incubator (37°C, 5% CO2, 95% air). After 3 days, non-adherent cells were removed by a single aspiration and medium was changed. Following another 4 days of culture, the adherent cells were harvested by incubation with trypsin/EDTA in PBS and gentle scraping with a rubber policeman. The number of cells was determined using a hemocytometer.

Evaluation of MFPC phenotype

MFPC phenotype was evaluated by indirect immunofluorescence staining for collagen type I (col I) and α-smooth muscle actin (αSMA). Adherent cells from two healthy control subjects and two patients with type 1 diabetes were cultured on fibronectin-coated eight-well glass chamber slides (Lab-Tek; Nunc, Naperville, IL, USA) at a density of 1×10^6 cells per well for 3, 7, and 17 days. Cells were fixed in ice-cold methanol/acetone and washed in PBS/0.05% Tween 20, followed by incubation with a monoclonal anticol I antibody (C2456; Sigma) at a dilution of 1:50 or a monoclonal anti-αSMA antibody (A2547, Sigma) at a dilution of 1:400. Binding of primary antibodies was detected by a tyramide signal amplification fluorescein technique, using horseradish peroxidase (HRP)-conjugated rabbit anti-mouse Ig (Dako, Glostrup, Denmark) followed by incubation with Fluorophore Tyramide Amplification Reagent (Perkin-Elmer, Boston, MA, USA). The

specificity of immunofluorescence staining was demonstrated by incubation with isotype-matched IgG control antibodies. Cells were counterstained with propidium iodide, mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA), and visualized by confocal laser scanning microscopy.
To confirm that the cultured MFPC were different from EPC, adherent cells were also incubated with 1,1-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled acetylated LDL (DiI-acLDL; Molecular Probes, Leiden, The Netherlands) for 1 h, and after fixation were incubated with fluorescein isothiocyanate (FITC)-labeled *Ulex europaeus* agglutinin I (*Ulex* lectin; Sigma) for 1 h. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and cells were visualized by fluorescence microscopy.

Analysis of MFPC proliferation and apoptosis
To measure cumulative MFPC proliferation in control subjects and diabetic patients, bromodeoxyuridine (BrdU; Sigma) was added to chamber slides of three control- and three diabetes-derived MFPC cultures at the time of seeding and each time when medium was changed (t=3, 7, 10, and 14 days). After 3, 7, and 17 days, cells were fixed in ice-cold ethanol and incubated with proteinase K. Fixed cells were treated with 4 N HCl, blocked with PBS/2% BSA/1% Triton X-100, and incubated with mouse-anti-BrdU (BD Biosciences, San Jose, CA, USA) at a dilution of 1:40. BrdU-positive cells were identified by incubation with HRP-conjugated rabbit-anti-mouse immunoglobulin (Dako) and enzyme activity of HRP was detected using 3,3′-diaminobenzidine (Sigma). Similarly, apoptotic activity of MFPC was analyzed by immunocytochemical staining with a rabbit anti-active caspase 3 antibody (BD Biosciences) at a dilution of 1:100. Both cumulative proliferation and apoptotic activity were assessed by counting positive stained nuclei.

Comparative gene expression profiling of MFPC
To determine gene expression profiles of MFPC from patients with diabetes compared with those of MFPC from healthy individuals, total RNA was extracted from 7-day cultured MFPC using RNeasy columns (Qiagen, Venlo, The Netherlands). The quantity and quality of the RNA samples were determined using a spectrophotometer (Shimadzu Biotech, Kyoto, Japan) with a Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) using the Eukaryote Total RNA Nano assay. Total RNA (300 ng) from diabetic patients and control subjects was pooled for each group in equal amounts for each subject. RNA pools were subsequently amplified using the MessageAmp Aminoallyl kit (Ambion, Austin, TX, USA) and labeled with Cy3 and Cy5 (Amersham Biosciences, Amersham, UK).
Duplicate dye-swap microarray hybridizations (i.e. a total of four hybridizations) with 300 ng of probe per sample were performed on 19 K human 70-mer oligochips (Genomics Laboratory, Utrecht, The Netherlands). Microarray slides were scanned using a DNA microarray scanner (Model G2565BA; Agilent Technologies) at 100% laser intensities and 30% photomultiplier tube sensitivity. Stored images were analyzed using Imagene (Biodiscovery, Marina Del Rey, CA, USA) software.
Data were normalized using the Lowess algorithm. Only spots with a signal intensity exceeding background +2-SD were considered. Log_2-transformed signal intensities between channels were normalized to have an identical log_2 median. For the present

report, genes were considered significantly regulated when in both experiments $\log_2$-transformed ratios were >0.7 or <−0.7 (equivalent to 1.62-fold up- and downregulation).

Quantitative PCR

BMP-6, BMP-7, and growth differentiation factor 9 (GDF-9) mRNAs were assessed in individual samples from diabetic patients and control subjects by quantitative real-time PCR using Assays-on-Demand Gene Expression Products with predesigned probe and primers (Applied Biosystems, Foster City, CA, USA). Hydroxymethylbilane synthase and TATA-box binding protein were used as internal references.

Individual mRNA expression is given as $\log_2$-transformed relative quantities adjusted to mean mRNA expression of the control group. (Relative $Q=2^{-\Delta\Delta Ct}$, $\Delta\Delta Ct=\Delta Ct[sample]-\Delta Ct[mean\ control]$, $\Delta Ct=Ct[target]-Ct[reference]$.)

BMP-6 and BMP-7 protein measurement

BMP-6 and BMP-7 protein levels were measured in conditioned media of three control- and three diabetes-derived MFPC cultures after 3, 7, and 17 days by commercial sandwich ELISA using a paired set of anti-human BMP-6 and BMP-7 antibodies, respectively (R&D Systems, Abingdon, UK). Standards were diluted in DMEM containing 20% fetal bovine serum. The detection limit of both assays was 50 pg/ml.

Culture of human renal fibroblasts

To study the effects of BMP on TGF-β activity, we used the SV40-transformed human renal fibroblast cell line TK173 (28). TK173 cells were maintained in DMEM supplemented with 10% fetal bovine serum, penicillin and streptomycin (Invitrogen). For gene expression experiments, cells were seeded at a density of 2×10^5 cells per well in six-well plates. After overnight culturing, cells were serum-starved for 12 h and stimulated with and without 1.5 ng/ml TGF-β1, in the presence or absence of 50 ng/ml BMP-6 and 200 ng/ml BMP-7 (all purchased from R&D Systems).

Effective concentrations of BMP-6 and BMP-7 were determined by readout of luciferase activity in TK173 cells transfected with a specific BMP responsive element construct coupled to a luciferase reporter. The observed EC50 values were similar to those provided in the data sheet by the manufacturer. After 4 h, total RNA was extracted, cDNA was synthesized, and gene expression of PAI-1 and CTGF was determined by quantitative PCR using Assays-on-Demand Gene Expression Products (Applied Biosystems), as described above.

Statistical analysis

Differences in MFPC number, proliferation, apoptosis, BMP-6 secretion, and mRNA expression were evaluated with Student's *t*-test or ANOVA with Bonferroni correction for multiple comparisons. Correlation between relative BMP-6 mRNA expression and the number of MFPC was assessed by linear regression. For all comparisons, a value of $P<0.05$ was considered to be significant (two-tailed).

Results

A subset of PBMC cultured on fibronectin develops into myofibroblasts

In both control- and diabetes-derived cultures, adherent cells at day 3 were all negative for col I and αSMA, which are markers of the myofibroblast phenotype (data not shown). After 7 days of culture, most of the adherent cells had obtained a spindle-shaped, fibroblast-like morphology (Figure 1A). In contrast to EPC cultures, which are known to be virtually all positive for uptake of DiI-acLDL and binding of *Ulex* lectin, MFPC cultures contained only a small number (<5%) of acLDL$^+$-Ulex lectin$^+$ cells (Figure 1B).

At day 7, approximately 70% of the control-derived adherent cells showed positive staining for col I and αSMA. After culture for an additional 10 days, virtually all adherent cells stained strongly positive for both col I and αSMA. In diabetes-derived MFPC cultures, cells at day 7 were more elongated than those in control-derived MFPC cultures, and approximately 90% were positive for col I and αSMA staining, compared with 70% in control-derived MFPC cultures. After 17 days of culture, all diabetes-derived adherent cells showed a myofibroblast phenotype (Figure 1C-F).

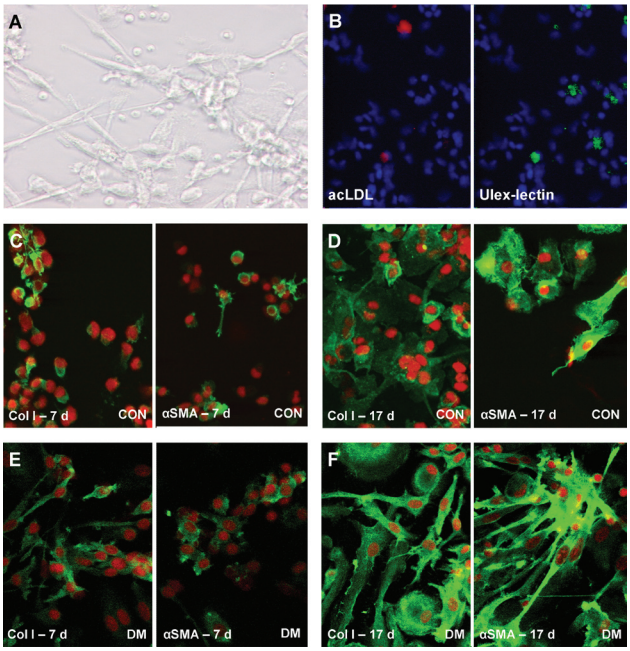

Figure 1. Outgrowth of MFPC from peripheral blood mononuclear cells. Mononuclear cells were isolated from peripheral blood and seeded on fibronectin-coated wells. A: After 7 days, approximately 70% of the adherent cells had obtained a fibroblast-like morphology. B: Fluorescence microscopy of MFPC with DAPI as nuclear stain (blue) illustrates that only a small number of adherent cells were positive for uptake of acLDL (red) and binding of *Ulex* lectin (green). C and D: Confocal laser scanning microscopy of control-derived MFPC with propidium iodide as nuclear stain (red) demonstrated that approximately 70% of adherent cells stained positive for col I and αSMA after 7 days. After 17 days, virtually all control-derived adherent cells showed a myofibroblast phenotype. E and F: In diabetes-derived MFPC cultures, approximately 90% of cells were positive for col I and αSMA after 7 days. After 17 days, all diabetes-derived adherent cells showed a myofibroblast phenotype.

The number of MFPC is increased in patients with diabetes

At day 7 of culture, the number of outgrowing MFPC per ml blood was significantly higher in patients with type 1 diabetes than in normoglycemic control subjects ($7.3\pm1.4\times10^4$ vs. $4.5\pm2.2\times10^4$, P=0.007; Figure 2A). This difference was also significant if the number of outgrowing MFPC was expressed relative to the number of initially seeded PBMC (7.5±1.2% in diabetic patients vs. 4.1±2.2% in control subjects, P=0.008).

To explore the possible contribution of proliferation and apoptosis to difference in MFPC numbers, three additional control- and diabetes-derived MFPC cultures were analyzed after 3, 7, and 17 days. After 7 days, all three diabetes-derived cultures contained higher MFPC numbers per ml blood than those from the three control subjects ($9.7\pm4.1\times10^4$ vs. $4.2\pm0.9\times10^4$; Figure 2B). These observations were within the range of the initial experiment (significance level of accumulated data, P=0.003).

The number of adherent cells in patients with diabetes tended to be higher already at day 3 of culture ($37.2\pm14.7\times10^4$ vs. $20.7\pm6.4\times10^4$, P=0.16), although this was not significant with this low number of observations (n=3 per group). Cumulative proliferation of MFPC was significantly higher in patients with diabetes after 7 days (3.5±0.3 vs. 0.5±0.3%, P=0.01) and 17 days (6.7±0.2 vs. 4.0±1.0%, P=0.04; Figure 2C), while apoptotic activity was significantly lower in diabetes-derived MFPC after 3 days of culture (31.0±1.5 vs. 43.2±2.7%, P=0.03; Figure 2D).

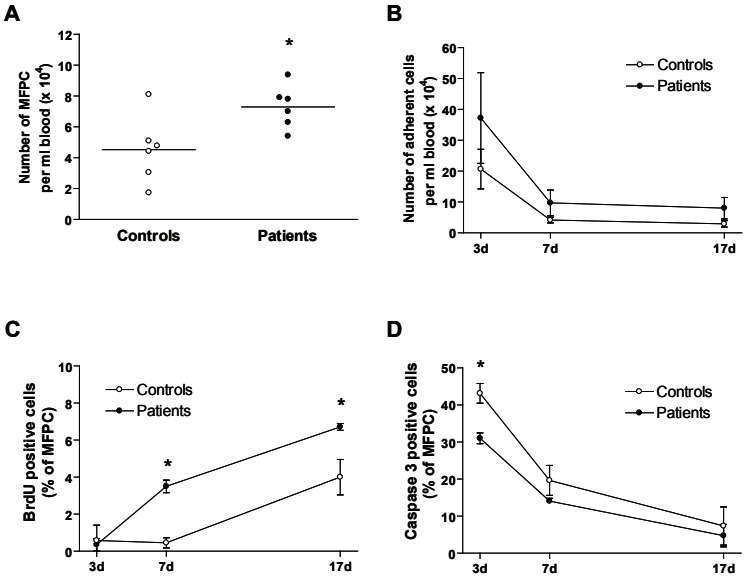

Figure 2. The number of MFPC in patients with diabetes and healthy controls. A: At day 7 of culture, the number of MFPC per ml blood was significantly higher in six patients with diabetes than in six age- and sex-matched control subjects. B: The time course (days 3, 7, and 17) of six additional cultures showed consistently higher numbers of MFPC in diabetic patients (n=3) than in control subjects. C: Cumulative proliferation of MFPC was significantly higher in diabetes-derived cultures. D: Apoptotic activity was significantly lower in diabetes-derived cultures. *P<0.05 for diabetes- vs. control-derived cultures.

BMP-6 expression is reduced in diabetes-derived MFPC

Of the genes present on the 19 K oligochip, those involved in TGF-β/BMP signaling pathways, including receptors, intra- and extracellular modulators, downstream mediators, transcription factors, and known targets of TGF-β and BMP signaling, were identified by searching PubMed (http://www.ncbi.nlm.nih.gov) and the Biomolecular Interaction Network Database (BIND; http://www.bind.ca) (29−31). Of all 426 genes thus identified, only BMP-6 and GDF-9 proved to be significantly regulated in both experiments. In MFPC derived from patients with diabetes compared with MFPCs derived from control subjects, both BMP-6 and GDF-9 showed a mean downregulation of 1.7-fold.

To validate the microarray data, BMP-6 and GDF-9 mRNA levels were assessed by quantitative PCR. In pooled, amplified RNA samples, quantitative PCR showed 2.7-fold downregulation of BMP-6 and 2.6-fold downregulation of GDF-9 in MFPC from patients with diabetes compared with control subjects. However, in samples from individual patients and control subjects, only BMP-6 mRNA expression proved to be significantly downregulated in diabetes-derived MFPC (3.9-fold reduction, P=0.007; Figure 3A), while mRNA expression of GDF-9 was particularly variable in both groups and not significantly different (P=0.24; data not shown).

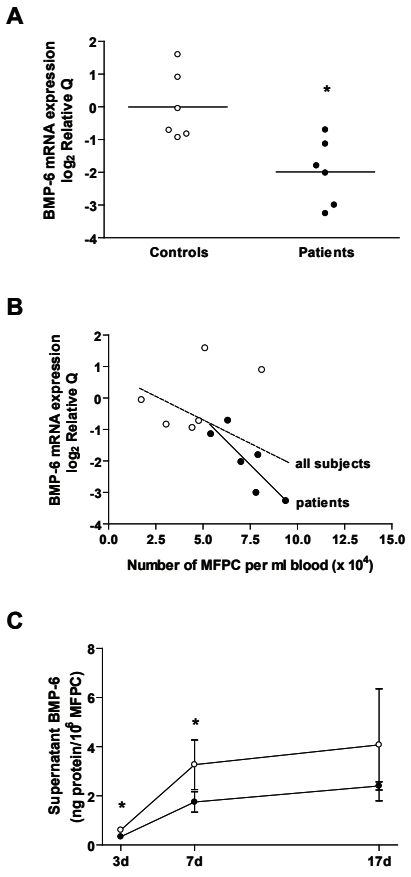

Figure 3. BMP-6 mRNA and protein expression in MFPC. A: BMP-6 mRNA expression in individual patients and control subjects was assessed by quantitative PCR and expressed as $\log_2$-transformed relative quantities adjusted to the mean BMP-6 mRNA expression of the control group (Relative Q). In patients with diabetes, $\log_2$ Relative Q was significantly lower than in control subjects. B: Separate analysis of data from diabetes-derived MFPC alone revealed a significant inverse correlation between BMP-6 mRNA level and the number of outgrowing MFPC (solid line; R=−0.85, P<0.05). When these parameters were analyzed in diabetic patients and control subjects together, a similar trend was observed, but this did not reach statistical significance (dashed line; R=−0.48, P=0.11). C: BMP-6 protein in conditioned medium was assessed by sandwich ELISA. Supernatant from diabetes-derived MFPC contained significantly less BMP-6 per 10^6 MFPC than supernatant from control-derived MFPC. Results are expressed as means±SD. *P<0.05 for diabetes- vs. control-derived MFPC.

Separate analysis of data from only diabetes-derived MFPC revealed a significant inverse correlation between BMP-6 mRNA level and the number of outgrowing MFPC (R=−0.85, P=0.03; Figure 3B). When these parameters were analyzed in diabetic patients and control subjects together, a similar trend was observed, but this did not reach statistical significance (R=−0.48, P=0.11). Measurement of BMP-6 protein in culture supernatants by ELISA revealed that diabetes-derived MFPC conditioned media contained approximately 45% less BMP-6 protein per 10^6 cells than supernatants of control-derived MFPCs (P=0.04; Figure 3C). BMP-7 was below the detection limit of microarray, quantitative PCR and ELISA.

BMP-6 inhibits TGF-β-induced target gene expression in human renal fibroblasts
Treatment of TK173 cells with 1.5 ng/ml TFG-β resulted in a 7.3-fold increase in PAI-1 (also known as SERPINE1) mRNA expression and a 2.5-fold increase in CTGF mRNA expression. Addition of BMP-6 (50 ng/ml) or BMP-7 (200 ng/ml) equally reduced PAI-1 and CTGF mRNA levels by approximately 70 and 50%, respectively (Figure 4).

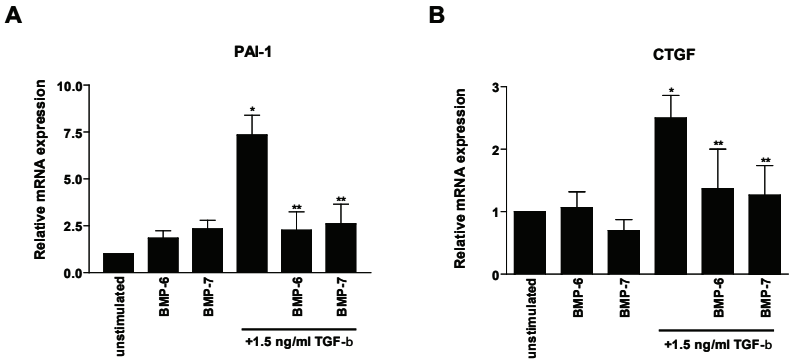

Figure 4. Effects of BMP-6 and BMP-7 on the TGF-β-induced expression of PAI-1 and CTGF mRNA in renal fibroblasts. TK173 cells were cultured in serum-free medium, and stimulated with and without TGF-β1 (1.5 ng/ml) in the presence and absence of BMP-6 (50 ng/ml) and BMP-7 (200 ng/ml). After 4 h of stimulation, total RNA was isolated and reverse-transcribed into cDNA for measurement of PAI-1 and CTGF levels by quantitative PCR. Treatment with TFG-β resulted in a 7.3-fold increase in PAI-1 mRNA expression (A) and a 2.5-fold increase in CTGF mRNA expression (B). Addition of BMP-6 or BMP-7 equally reduced PAI-1 (by 70%) and CTGF mRNA levels (by 50%). Results are expressed as means±SD of triplicate experiments. *P<0.05 vs. unstimulated condition; **P<0.05 vs. stimulation with 1.5 ng/ml TGF-β.

Discussion

The two main findings of the present study are the increased number of outgrowing MFPC obtained from peripheral blood of patients with diabetes and the decrease in BMP-6 expression in diabetes-derived MFPC. Taking into account that diabetes- and control-derived MFPC were cultured under identical conditions in the presence of 20% fetal bovine serum, these are remarkable findings, which suggest robust alternative programming of cPC in these diabetic patients.
It would be interesting to know whether an increased number and altered gene expression

of MFPC in diabetes is already evident in freshly drawn blood. However, in contrast to EPC, which express CD34, CD133, and kinase insert domain receptor (KDR) in peripheral blood and could therefore be isolated directly from the circulation (32), no such specific markers are known to exist for circulating MFPC. Thus far, the only way to obtain MFPC is by *ex vivo* cell culture. Hence, it is not possible to analyze the number of MFPC in freshly drawn blood.

The increased number of MFPC in cultures derived from patients with diabetes might be related, at least in part, to the lower apoptotic activity and increased proliferation observed in diabetes-derived MFPC cultures. Interestingly, a reduced proliferation rate and augmented apoptotic activity has been observed in cultures of control-derived EPC under hyperglycemic conditions (33), which could explain the remarkable observation of increased MFPC in contrast to decreased EPC in diabetes. The precise origin of MFPC is still unclear. We observed that MFPC were negative for acLDL uptake and *Ulex* lectin staining. Interestingly, *in vitro* EPC, which are acLDL^{+}–Ulex lectin^{+} cells, can be derived from monocytes/macrophages (34). Whether MFPC can also be derived from monocytes/macrophages remains to be established.

The differential gene expression in diabetes- vs. control-derived MFPC suggests robust programming in patients with diabetes, since it appeared to be resistant to the expected equalizing effect of prolonged culture under identical conditions in 20% fetal bovine serum. Similarly, prolonged maintenance of aberrant function/gene expression in patients with diabetes has been observed in EPC cultures (12) and in cultured skin fibroblasts (35). Furthermore, others have shown that bone marrow-derived mesangial cell progenitors are able to transmit a disease phenotype to normal glomeruli (36) and also play a crucial role in the development of diabetic nephropathy (37).

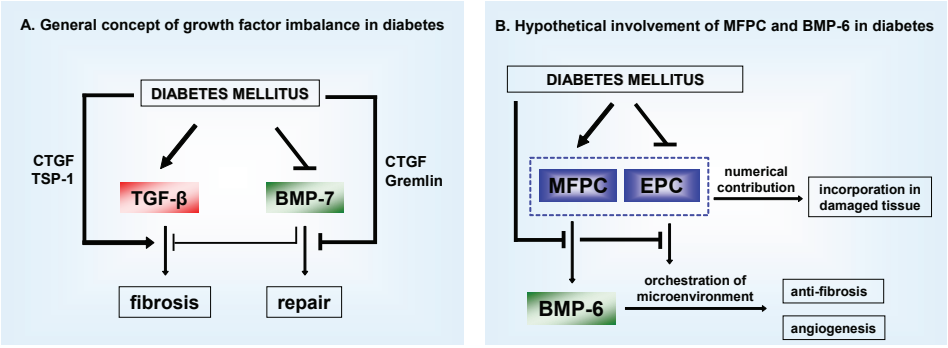

Figure 5. Concept of growth factor imbalance affecting the contribution of MFPC in diabetes. A: Increased TGF-β signaling in diabetes, which is amplified by factors such as CTGF and thrombospondin-1 (TSP-1), results in fibrosis, while repair is hindered by the downregulation of BMP-7 signaling, as well as the diabetes-induced BMP antagonists CTGF and gremlin. B: The number of EPC is decreased in diabetes, whereas the number of MFPC is increased, along with the downregulation of BMP-6 expression. In view of the antifibrotic and pro-angiogenic effects of BMP-6, we propose that the contribution of BMP-6-deficient MFPC in diabetes might involve inappropriate orchestration of resident cells in their microenvironment, in addition to fibroplasia due to increased numbers of infiltrating MFPC *per se*.

The origin and nature of aberrant programming of gene expression in diabetes is not known. One possibility is that this relates to growth factor imbalance *in vivo*, which is also involved in the misdirection of the tissue response to injury in the diabetic condition. In particular, TGF-β activity is increased in diabetes, whereas the BMP-7 pathway is downregulated (Figure 5A). The increased TGF-β production and signaling, which is further amplified by, for example, CTGF and thrombospondin-1, results in fibrosis (13;15;22;38), while the maintenance and repair of functional tissue is hindered by the downregulation of BMP-7 signaling, which is due to reduction of ligand and receptor expression by the diabetic condition, as well as to the diabetes-induced BMP antagonists CTGF and gremlin (24;39).

To examine whether gene expression in diabetes-derived MFPC is indicative of TGF-β/BMP signaling imbalance, we performed microarray experiments followed by quantitative PCR validation. Data analysis was focused on genes related to TGF-β/BMP superfamily members, i.e. inducers, ligands, receptors, modulators, mediators, and targets. Of 426 genes identified, according to our analyses, only BMP-6 was significantly (down-)regulated in diabetes-derived MFPC. The observed downregulation of BMP-6, which was evident also at the protein level, is of particular interest because of the pivotal role of BMP-6 in angiogenesis (40). This is an interesting analogy with the release by EPC of potent pro-angiogenic growth factors (34), which supports the hypothesis that the *in vivo* angiogenic activity of EPCs may include a role in the orchestration of their microenvironment. The expression of the angiogenic factor BMP-6 by MFPC (which is reduced in diabetes) might suggest similar involvement of these cells in the tissue response to injury.

Although BMP-7 has received much attention as a renoprotective factor in diabetes and other conditions, no reports are available concerning a possible role of BMP-6 regulation in diabetes. However, BMP-6 and BMP-7 are structurally very similar and both act as specific ligands for the BMP-receptor activin receptor-like kinase-2 (ALK-2), unlike other BMPs (29). Furthermore, crosstalk between BMP-6 and TGF-β has been observed (41), and BMP-6 has the capacity to substitute for BMP-7 in kidney development (42). We demonstrate here for the first time that, like BMP-7, BMP-6 antagonizes profibrotic TGF-β activity by decreasing PAI-1 and CTGF expression in renal interstitial fibroblasts. We observed comparable antagonistic effects on TGF-β activity in mesangial cells and tubular epithelial cells (data not shown).
BMP-7 expression was below the detection limit of microarray, quantitative PCR, and ELISA in our MFPC cultures. These data suggest that, as far as MFPC are concerned, BMP-6 rather than BMP-7 expression is aberrantly regulated in diabetes. Interestingly, decreased BMP-6 expression has also been observed in (myo-)fibroblasts obtained from fibrotic Dupuytren's lesions compared with fibroblasts harvested from normal palmar fascia (43). BMP-7 expression in (myo-)fibroblasts was below the detection limit in that study, as was the case for MFPC in the present study. Since BMP-6 levels were still very low in culture supernatant of MFPC at day 3 (which had not yet obtained the myofibroblast phenotype), we propose that BMP-6 expression occurred mainly during or after the acquisition by the cells of a mesenchymal phenotype. In agreement with this notion, BMP-6 expression in the developing kidney is mainly present in loose mesenchymal

stromal cells, in contrast to BMP-7, which is expressed in epithelial cells (44).

Together with the known angiogenic effect of BMP-6 on endothelial cells, our novel observations suggest that the contribution of MFPC in diabetes might involve the inappropriate orchestration of resident cells in their microenvironment, in addition to fibroplasia due to increased numbers of infiltrating MFPC *per se* (Figure 5B). According to this concept, aberrant programming of cPC would act as a double-edged sword in the adverse remodeling of damaged tissue in diabetes.

Acknowledgments

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Chapter VII

BMP-6 Determines the Number of Circulating Myofibroblast Progenitor Cells and Attenuates Renal Fibrosis

TQ Nguyen[1], O van Oostrom[2], DM van der Giezen[1], JW Leeuwis[1], C Snijckers[1], JA Joles[3], EJ Robertson[4], MC Verhaar[2], and R Goldschmeding[1]

[1]Department of Pathology, University Medical Center Utrecht, The Netherlands
[2]Department of Vascular Medicine, University Medical Center Utrecht, The Netherlands
[3]Department of Nephrology, University Medical Center Utrecht, The Netherlands
[4]Sir William Dunn School of Pathology, University of Oxford, United Kingdom

Manuscript in preparation

Abstract

Introduction: Myofibroblast progenitor cells (MFPC), also known as fibrocytes or smooth muscle progenitor cells, are α-smooth muscle actin (αSMA) positive, collagen-secreting bone marrow-derived cells. Previously, we reported that the number of circulating MFPC from patients with diabetes was increased and that MFPC from these patients expressed less BMP-6 as compared to MFPC derived from control subjects. In the present study, we set out to assess the impact of BMP-6 deficiency on the course of renal fibrosis, in particular in relation to circulating MFPC.

Materials and methods: MFPC were cultured from mononuclear cells isolated from the spleen of BMP-6 null mice and wild-type mice. The number of circulating MFPC was determined by flow cytometry for αSMA. Unilateral ureteral obstruction (UUO) was performed by permanent ligation of the left ureter for 7 days. The presence of MFPC in renal tissue was determined by dual-positive staining for αSMA and leukocyte-specific protein-1. Extracellular matrix accumulation was assessed by Q-PCR for αSMA and collagen 1α2 mRNA. BMP target gene expression was determined by Id1, Id2, Id3, and Smad6 mRNA.

Results: Compared to wild-type mice, BMP-6 null mice had higher numbers of circulating MFPC, decreased renal cortical BMP target gene expression, and increased αSMA mRNA in the obstructed kidney. BMP-7 mRNA was similarly decreased in the obstructed kidney of wild-type mice and BMP-6 null mice, while BMP-6 mRNA was higher in the obstructed kidney of wild-type mice. In obstructed kidneys of BMP-6 null mice and wild-type mice, MCP-1 expression and macrophage influx was similar, and infiltration of MFPC in the tubulointerstitium was focal and very limited.

Conclusions: Our observations demonstrate that endogenous BMP-6 determines the number of circulating MFPC and that it attenuates renal fibrosis. These data indicate that BMP-6, in addition to the known renoprotective effects of BMP-7, might be another important factor in regeneration of the adult kidney.

Introduction

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor (TGF)-β superfamily, which consists of more than thirty growth factors including inhibins, activins, and the three isoforms of TGF-β. BMPs are essential in development, throughout which they regulate cell growth, survival and differentiation, morphogenesis, and general organogenesis. In the kidney, the importance of several BMPs has been demonstrated mainly in developmental studies (1–3). Thus far, only BMP-7 has also been studied for its contribution as an antifibrotic and proregenerative factor in response to injury of the adult kidney. Renal expression of BMP-7 is progressively decreased during the course of renal fibrosis, while restoration of BMP-7 availability has resulted in prevention or even reversal of functional and structural changes (4–6). Although BMPs other than BMP-7 are also expressed in the adult kidney, little is known about their particular function in homeostasis of the postnatal kidney (7).

Myofibroblast progenitor cells (MFPC), also known as fibrocytes or smooth muscle progenitor cells, are collagen-secreting bone marrow-derived cells which stain positive for leukocyte-specific markers CD45 and leukocyte-specific protein (LSP)-1, and for collagen type I and α-smooth muscle actin (αSMA) (8–11). MFPC can be cultured from peripheral blood mononuclear cells. They contribute to fibrotic tissue remodeling, as has been shown in experimental and clinical conditions including asthma, hypertrophic scars, chronic allograft vasculopathy, and nephrogenic systemic fibrosis (10;12–14). Furthermore, MFPC are directly involved in renal fibrosis, and blockade of infiltrating MFPC was successful in reduction of extracellular matrix (ECM) accumulation induced by unilateral ureteral obstruction (UUO) (11).

Previously, we reported that the number of MFPC in peripheral blood from patients with diabetes was significantly increased as compared to normoglycemic control subjects (15). Moreover, this study demonstrated that MFPC derived from patients with diabetes had 3.9-fold less BMP-6 mRNA expression, and that BMP-6 could antagonize profibrotic activity of TGF-β1 in renal cells. The renoprotective effect of BMP-6 was also indicated by the observations that BMP-6 was the most effective member of the TGF-β superfamily in reducing the expression of proinflammatory genes in proximal tubular epithelial cells (16), and that BMP-6 mRNA was downregulated in kidneys from mice with diabetic nephropathy and from rats with hypertensive renal damage (7;17).

In the present study, we set out to assess the impact of BMP-6 on the number of circulating MFPC, and their contribution to renal fibrosis in a mouse model of chronic obstructive nephropathy.

Materials and Methods

Animals

Outbred male C57Bl/6J x 129Sv BMP-6 null mice, in which the 3' end of exon 2 of the BMP-6 gene was deleted by insertion of a neomycin resistance gene (18), were mated with female C57Bl/6J mice. From their offspring, female BMP-6 null mice and female wild-type mice were used for the present study. All mice were housed in standard cages in

a room with constant temperature, on a 12-h light-dark cycle. Animals were fed a standard pellet laboratory chow and had free access to water. The experiments were performed with the approval of the Experimental Animal Ethics Committee of the University of Utrecht.

Culture of myofibroblast progenitor cells

MFPC were cultured as described previously (15), according to protocols adopted from Bucala and others for culture of fibrocytes, i.e. collagen-secreting cells cultured from peripheral blood mononuclear cells (8;19). Mononuclear cells (MNC) were isolated from the spleen of BMP-6 null mice (n=6) and wild-type mice (n=6), and plated on fibronectin-coated dishes in Dulbecco's Modified Eagle Medium supplemented with 20% heat-inactivated fetal bovine serum, and antibiotics (penicillin 100U/ml and streptomycin 100µg/ml; Invitrogen, Carlsbad, CA, USA). After 4 days in culture, cells were detached using trypsin-EDTA and a cell scraper. MFPC were identified by staining for αSMA-Phycoerythrin (R&D systems, Abingdon, UK) and counted by flow cytometry (Beckman Coulter, Fullerton, CA, USA). Isotype-stained samples served as negative controls.

Unilateral ureteral obstruction

UUO was induced in BMP-6 null mice and wild-type mice (n=11 per group). From both groups, 6 sham-operated mice were used as controls. Mice were anesthetized by inhalation of isoflurane. After a small incision was made on the flank, the left ureter was permanently ligated at two points. Mice were killed 7 days after surgery. Renal cortex was harvested by dissecting small caps of the upper and the lower poles. Before homogenization, absence of medulla was checked in frozen sections of the cut surface. Plasma creatinine was measured with an enzymatic assay (J2L Elitech, Labarthe Inard, France). Albuminuria was determined by sandwich ELISA (Bethyl Laboratories, Inc., Montgomery, TX, USA).

Renal histology and immunohistochemistry

Renal morphology was assessed by staining paraffin-embedded sections with periodic acid Schiff (PAS). Infiltration of macrophages was determined in frozen kidney sections, which were fixed with acetone, blocked, and incubated with a rat antibody against the mouse macrophage antigen F4/80 (Serotec Benelux, Oxford, UK). Sections were then incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-rat (Dako, Glostrup, Denmark) and goat anti-rabbit Powervision-HRP (Klinipath, Duiven, The Netherlands), developed with Nova Red (Vector Laboratories, Burlingame, CA, USA) and counterstained with hematoxylin. The number of F4/80 positive cells per high power field (field area 0.245 mm^2) was assessed in a blinded fashion.

The presence of MFPC in the kidney was evaluated by double immunofluorescence staining for LSP-1 and αSMA. Paraffin-embedded sections were incubated with a mouse LSP-1 specific rabbit polyclonal antibody (20) (kindly provided by Jan Jongstra, Toronto, Canada), followed by incubation with TRITC-labeled swine anti-rabbit IgG (Dako). Subsequently, slides were incubated with a mouse-anti-αSMA antibody (Sigma, St Louis, MO, USA), which was biotinylated using the ARK Kit (Dako), streptavidin-HRP, FITC-labeled rabbit anti-streptavidin (Vector Laboratories), and amplified with

Fluorophore Tyramide Amplification Reagent (Perkin-Elmer, Boston, MA, USA). Slides were counterstained with TOPRO-3, mounted in Vectashield (Vector Laboratories), and visualized by confocal laser scanning microscopy.

Quantitative PCR

Total RNA was extracted from 30 mg frozen renal cortex using RNeasy columns (Qiagen, Venlo, The Netherlands). After cDNA synthesis, expression of αSMA (ACTA2), collagen type 1α2 (COL1A2), monocyte chemotactic protein (MCP)-1, BMP-6, BMP-7, Id1, Id2, Id3, and Smad6 mRNA was assessed by quantitative real-time PCR using TaqMan Gene Expression Assays with pre-designed probe and primers (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal reference.

Statistical analysis

Data are presented as mean±SEM. Differences between groups were analyzed by Student's *t*-test or ANOVA with Bonferroni correction for multiple comparisons. For all comparisons, a value of $P<0.05$ was considered to be significant (two-tailed).

Results

BMP-6 deficiency increases the number of MFPC

The renal phenotype of BMP-6 null mice was comparable to that of wild-type mice. No significant differences were observed regarding glomerular size and density, kidney weight, kidney/body weight, albuminuria, and plasma creatinine (data not shown).

MFPC were cultured from the spleen of BMP-6 null mice and wild-type mice. The number of MNC at t=0 isolated from the spleen was not different between wild-type mice and BMP-6 null mice ($75.0\pm3.0\times10^6$ vs. $74.5\pm26.7\times10^6$; Figure 1A). After 4 days of culture, the number of total viable and adherent MNC tended to be higher in BMP-6 null mice than in wild-type mice, but this was not statistically significant ($81.9\pm40.5\times10^6$ vs. $29.0\pm6.9\times10^6$; Figure 1B). After 4 days of culture, the number of αSMA positive MFPC was 2.4-fold higher in BMP-6 null mice than in wild-type mice (47±7% vs. 19±4% of adherent cells, P=0.008; Figure 1C).

BMP-6 deficiency is associated with decreased BMP target gene expression

The expression of BMP-target genes Id1, Id2, and Id3 were significantly lower in BMP-6 null mice as compared to wild-type mice, both in the obstructed kidney and in the contralateral kidney. Also the expression of BMP-target gene Smad6 was lower in BMP-6 null mice, but this was only significant in the obstructed kidney and not in the contralateral kidney (P=0.059). Interestingly, mRNA levels of Id2, Id3, and Smad6 were all increased in the obstructed kidney of both wild-type mice and BMP-6 null mice, while expression of Id1 was not different after ureteral obstruction (Figure 2A-D).

The expression of BMP-6 mRNA was 2.6-fold increased in the obstructed kidney of wild-type mice, while the expression of BMP-7 was decreased similarly in the obstructed kidney of both BMP-6 null mice and wild-type mice (Figure 2E and 2F).

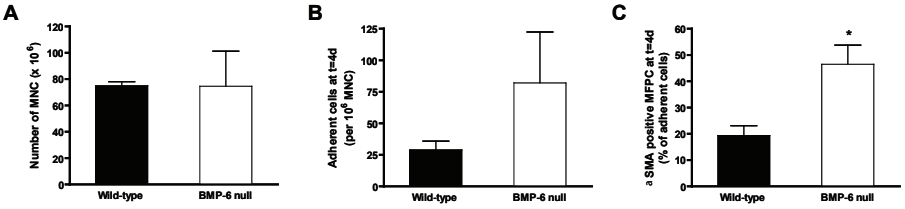

Figure 1. Outgrowth of MFPC in wild-type mice and BMP-6 null mice. MFPC were obtained by culture of spleen MNC for 4 days and counted by flow cytometry for αSMA. A: The number of MNC isolated from the spleen was not different between wild-type mice and BMP-6 null mice. B: After 4 days of culture, the number of adherent cells tended to be higher in BMP-6 null mice than in wild-type mice. C: After 4 days of culture, the number of αSMA positive cells was 2.4-fold increased in BMP-6 null mice. Data are expressed as mean±SEM. *P<0.05 vs. wild-type mice.

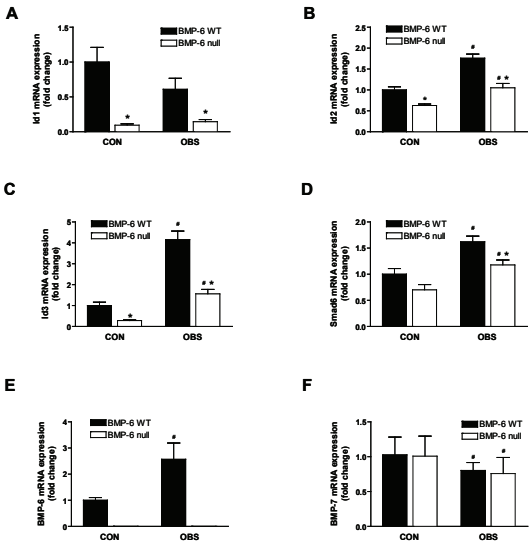

Figure 2. Renal expression of BMP target genes, BMP-6, and BMP-7 in wild-type and BMP-6 null mice. Renal cortex was harvested 7 days after UUO. A-D: Expression of Id1, Id2, Id3, and Smad6 mRNA was decreased in BMP-6 null mice as compared to wild-type mice. E: BMP-6 mRNA was increased in the obstructed kidney of wild-type mice. F: BMP-7 mRNA was decreased similarly in the obstructed kidney of wild-type and BMP-6 null mice. Data are expressed as mean±SEM. *P<0.05 versus wild-type mice, #P<0.05 versus contralateral kidney.

BMP-6-deficiency increases renal fibrosis, but not macrophage influx

ECM accumulation was determined by gene expression for αSMA and procollagen type 1α2. After 7 days of UUO, no significant increase in αSMA expression was observed in the obstructed kidney of wild-type mice as compared to the contralateral kidney. In contrast, αSMA expression was significantly increased in the obstructed kidney of BMP-6 null mice, and this increase was 2.0-fold higher than in the obstructed kidney of wild-type mice (Figure 3A). Expression of procollagen type Iα2 was significantly increased in the obstructed kidney of both wild-type mice and BMP-6 null mice, and it tended to be higher in the obstructed kidney of BMP-6 than the obstructed kidney of wild-type mice (1.5-fold change), but this did not reach statistical significance (P=0.14; Figure 3B).

The expression of MCP-1 mRNA in both wild-type and BMP-6 null mice was significantly increased in the obstructed kidney as compared to the contralateral kidney, but no

difference was observed between both groups (Figure 3C). Concordantly, the number of infiltrating F4/80 positive macrophages in the renal cortex was not different between wild-type and BMP-6 null mice (Figure 3D).

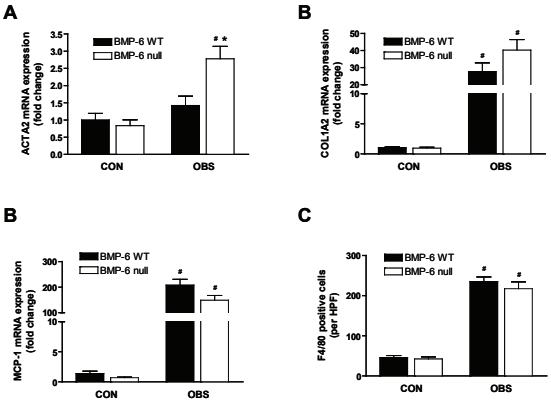

Figure 3. ECM accumulation and macrophage infiltration in the kidney of wild-type and BMP-6 null mice. Renal cortex was harvested 7 days after UUO. A: Expression of αSMA mRNA was significantly increased in the obstructed kidney of BMP-6 null mice as compared to wild-type mice. B: Increase of collagen type Iα2 mRNA tended to be higher in the obstructed kidney of BMP-6 null mice. C-D: No difference in MCP-1 mRNA and influx of F4/80 positive macrophages was observed between BMP-6 null mice and wild-type mice. Data are expressed as mean±SEM. *P<0.05 vs. wild-type mice, #P<0.05 vs. contralateral kidney.

Very limited numbers of infiltrating MFPC in obstructed kidney of wild-type mice and BMP-6 null mice

Infiltration of MFPC in renal tissue was assessed by double immunofluorescence staining for LSP-1 and αSMA. In sham-operated kidneys and contralateral kidneys from wild-type and BMP-6 null mice, no dual positive cells were observed (data not shown). In contrast, dual positive cells were present in the tubulointerstitium of obstructed kidneys (Figure 4). However, the presence of these cells was focal and very limited, and no clear difference in infiltrating MFPC was observed between BMP-6 null mice and wild-type mice.

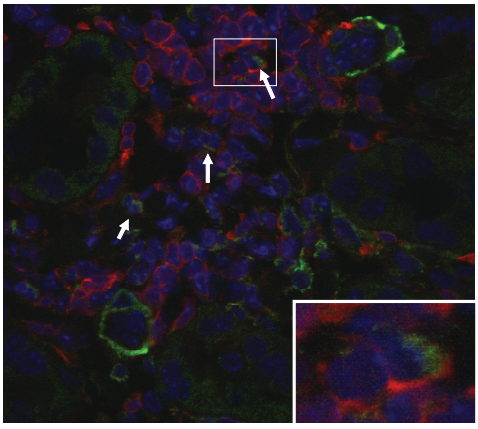

Figure 4. Infiltration of MFPC in the obstructed kidney of BMP-6 null mice. Infiltration of MFPC in renal tissue was assessed by double immunofluorescence staining for LSP-1 (red) and αSMA (green). Nuclei were counterstained with TOPRO-3 (blue). Very few dual positive cells were focally present in the tubulointerstitium of the obstructed kidney (see arrows and inset). No clear difference in infiltrating MFPC was observed between BMP-6 null mice and wild-type mice.

Discussion

Previously, we reported that higher numbers of circulating MFPC were associated with lower expression of BMP-6 in patients with type 1 diabetes (15). Here, we complement this finding with the observation that BMP-6 deficiency *per se* is sufficient for increased outgrowth of MFPC from the spleen, and that BMP-6 deficiency aggravates renal fibrosis in the UUO model.

During kidney development, expression of BMP-6 is observed in discrete stromal cells near the ureteric tree (21). However, the precise role of BMP-6 in renal development is not clear. BMP-6 null mice do not show kidney abnormalities at birth (18), and renal structure and function in adult BMP-6 null mice were also normal in the present study.
Nothing is known about the biological functions of BMP-6 in the adult kidney. Considering their high homology, one might expect that, like BMP-7, BMP-6 is involved in homeostasis and regeneration of the adult kidney. This is underscored by previous *in vitro* observations that BMP-6 was the most effective member of the TGF-β superfamily in reducing the expression of proinflammatory genes in proximal tubular epithelial cells (16), and that BMP-6 antagonizes profibrotic activity of TGF-β1 in renal interstitial fibroblasts (15). In the present study, we demonstrated that BMP-6 deficiency *in vivo* was directly associated with aggravation of renal fibrosis induced by UUO, as exemplified by increased αSMA mRNA expression in the obstructed kidney of BMP-6 null mice. This effect was independent of BMP-7, of which the expression in the obstructed kidney was similarly decreased in wild-type mice and BMP-6 null mice.
Members of the TGF-β superfamily, including all BMPs, signal through binding to serine/threonine kinase receptors and phosphorylation of Smads. Upon translocation of activated Smads into the nucleus, specific target genes are transcribed, of which Id1, Id2, Id3, and Smad6 are mainly induced by BMPs (22). Thus, despite the existence of more than twenty BMP family members in mammals, target gene expression of the different BMPs converges at the receptor level. Interestingly, BMP target gene expression in BMP-6 null mice was consistently decreased in BMP-6 null mice as compared to wild-type mice, both in the obstructed kidney and in the contralateral kidney. This indicates that BMP target gene expression in the adult kidney is significantly determined by endogenous BMP-6 expression level, and at least not exclusively by BMP-7. This hypothesis is also supported by the observation that expression of Id2, Id3, and Smad6 mRNA was significantly higher in the obstructed kidney than in the contralateral kidney at day 7, in parallel with the expression level of BMP-6, but not BMP-7. However, analysis of BMP ligand expression in relation to BMP target gene expression at more time points will be needed in order to clarify the role of different BMPs during development and progression of renal fibrosis.

The exact mechanism by which BMP-6 attenuates renal fibrosis remains to be established. Renal injury is known to be associated with infiltration of inflammatory cells including macrophages (23). However, no relation between BMP-6 deficiency and MCP-1 expression and macrophage infiltration was observed in the present study. Also the expression of connective tissue growth factor and plasminogen activator inhibitor-1, both target genes of TGF-β1, was not higher in the obstructed kidney of BMP-6 null mice as

compared to wild-type mice (data not shown), suggesting that BMP-6 did not directly influence profibrotic TGF-β activity *in vivo*.
Although BMP-6 deficiency was associated with a higher number of circulating MFPC in our study, infiltration of MFPC in the obstructed kidney appeared to be extremely limited in both wild-type mice and BMP-6 null mice. This is in contrast to other reports, in which the number of infiltrating MFPC in the obstructed kidney was high (average of 19.2 cells per mm^2), and maximal at day 7 after UUO (11;24). However, infiltration of MFPC in those studies was assessed by double immunofluorescence for CD45 and collagen type I, while MFPC in our study were identified by LSP-1 and αSMA dual-positive cells. Since the total number of αSMA positive interstitial cells in our study was low at day 7 after UUO, we might not have identified MFPC that do not yet express αSMA protein at this time point. To resolve this issue, we are currently assessing the number of infiltrating MFPC by additional collagen type I staining.
Existing data concerning the contribution of MFPC to renal fibrosis are contradictory. Although several papers have reported substantial infiltration of functional MFPC in the kidney after UUO in mice and ischemia/reperfusion injury in rats (11;24;25), others have demonstrated that bone-marrow derived cells do not contribute significantly to collagen I synthesis in renal fibrosis (26), and that enhanced mobilization of bone-marrow derived cells does not have an impact on development of renal fibrosis (27). In our study, renal fibrosis was aggravated in BMP-6 null mice, without evident increase in the number of infiltrating MFPC in the kidney. However, we cannot exclude that BMP-6 deficient MFPC might contribute to renal fibrosis also by inappropriate orchestration of resident renal cells, e.g. via aberrant secretion of paracrine cytokines and growth factors.

In conclusion, we have demonstrated that BMP-6 determines the number of myofibroblast progenitor cells and that it attenuates renal fibrosis after induction of UUO. However, the mechanism by which this occurs remains unknown and further studies are needed to elucidate the direct impact of BMP-6 deficient MFPC on renal fibrosis.

Acknowledgments

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Chapter VIII

BMP-7 and CTGF: Novel Targets for Treatment of Renal Fibrosis?

TQ Nguyen and R Goldschmeding

Department of Pathology, University Medical Center Utrecht, The Netherlands

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Abstract

Renal fibrosis is the major determinant in progression of kidney disease and results from an inappropriate response to acute and chronic kidney injury. Transforming growth factor (TGF)-β1 is the driving force behind renal fibrosis and has since long been regarded as the key factor to be targeted in prevention and treatment of renal fibrosis. Despite the impressive results obtained in experimental renal fibrosis, TGF-β1 blockade has not yet translated into an effective and safe therapeutic in human patients. Therefore, it remains important to explore the role of additional growth factors which are involved in renal regeneration and fibrosis.

Recently, bone morphogenetic protein (BMP)-7 and connective tissue growth factor (CTGF) have both emerged as novel modulators of profibrotic TGF-β1 activity. The expression of BMP-7 is decreased in various models of renal disease, while CTGF is strongly upregulated in experimental and human renal fibrosis. In experimental kidney injury, administration of BMP-7 or inhibition of CTGF have been sufficient to result in striking improvement of renal function and structure.

This review summarizes the current knowledge of BMP-7 and CTGF in the kidney, and discusses their therapeutic potential in renal fibrosis.

Background

Renal fibrosis

Renal fibrosis is the major determinant in progression of kidney disease and results from a general set of responses to acute and chronic kidney injury, irrespective of the initial cause. Renal fibrosis is characterized by excessive accumulation of extracellular matrix (ECM) components leading to glomerulosclerosis, tubulointerstitial fibrosis, inflammatory infiltration, loss of renal parenchyma, and renal vascular changes. The origin of matrix production in the kidney is not completely understood, but might include activation of resident fibroblasts, epithelial and mesangial cells, migration of hematopoietic or mesenchymal stem cells from the bone marrow, migration of periadventitial cells, and epithelial-to-mesenchymal transition (EMT) of tubular epithelial cells (1;2).

Fibrosis, response to injury, and growth factors

Under physiological conditions, local damage or altered environmental factors will result in an appropriate response to injury of the affected kidney resulting in minimal scarring and optimal repair and functional recovery. However, in many instances, inappropriate response to injury occurs, favoring scar formation over repair. This contributes significantly to progressive renal fibrosis, and subsequently to end-stage renal failure.

The precise molecular mechanisms of renal fibrosis have not been fully elucidated, but renal response to injury is considered to be determined by the expression level of certain growth factors. Transforming growth factor (TGF)-β1 is generally regarded as the key mediator in the development of renal fibrosis (3). Although many therapeutic approaches have been explored to inhibit TGF-β1 activity in experimental models of renal disease, thus far no anti-TGF-β1 therapy has been applied in a clinical setting (4). Therefore, it remains important to explore the role of additional growth factors which are involved in renal regeneration and fibrosis. In this respect, bone morphogenetic protein (BMP)-7 and connective tissue growth factor (CTGF) might be attractive novel targets in the treatment of renal fibrosis.

TGF-β1 is the Driving Force Behind Renal Fibrosis

Transforming growth factor-β1 (TGF-β1)

TGF-β1 is a member of the TGF-β superfamily, which consists of more than 30 growth factors, including BMPs, inhibins, and activins (5). Members of the TGF-β superfamily signal through binding to type I and type II serine/threonine kinase receptors. Upon ligand binding and receptor complex activation, receptor regulated (R)-Smads are phosphorylated. Phophorylated R-Smads bind to their common partner Smad4, and subsequently translocate into the nucleus where they act as transcription factors. The specificity of the signal is mainly determined by the type I receptor, also known as activin receptor-like kinase (ALK), and the R-Smads. In general, TGF-βs bind ALK5 and activate Smad2 and -3, while BMPs bind ALK2, -3, and -6, and activate Smad1, -5, and -8 (6).

In mammals, three isoforms of TGF-β (TGF-β1, TGF-β2, and TGF-β3) have been identified, which are encoded by genes lying on separate chromosomes, but share a high

degree of homology. Since all TGF-β isoforms also show comparable *in vitro* biological activities and signal through the same set of receptors, it has been suggested that they have equal biological activity *in vivo*. However, studies with TGF-β isoform-specific knockout mice have revealed that each TGF-β isoform exerted specific and non-compensated functions, which might relate to their individual expression regulation and distribution (7). The effects of TGF-β1 are multifunctional and, in addition to its fibrogenic role as main inducer of ECM production, the functions of TGF-β1 also include regulation of cell growth, cell differentiation, cytokine production, and immune cell modulation (8–10).

TGF-β1 expression in renal fibrosis

The relevance of TGF-β1 in renal disease was first reported in a landmark study by Border *et al*., who provided direct evidence for a causal role of TGF-β1 in ECM accumulation in experimental acute mesangial proliferative glomerulonephritis (11). Subsequently, upregulation of TGF-β1 expression has been demonstrated in a wide variety of other experimental diseases associated with renal fibrosis, including anti-glomerular basement membrane disease, obstructive nephropathy, hypertensive nephrosclerosis, and diabetic nephropathy (12–15). In human renal biopsies, upregulation of TGF-β1 and isoforms was observed in glomerular and tubulointerstitial diseases characterized by ECM accumulation, but not in renal disorders where ECM accumulation was not a feature (15–17). In addition, levels of circulating and/or urinary TGF-β1 protein were shown to be elevated in patients with renal fibrotic diseases like chronic allograft nephropathy, focal segmental glomerulosclerosis, and diabetic nephropathy (18–20).

Inhibition of TGF-β1 activity

Several therapeutic strategies have been applied successfully to inhibit profibrotic TGF-β1 activity in experimental models of renal fibrosis. These approaches include inhibition of TGF-β1 by neutralizing antibodies, antisense oligodeoxynucleotides, soluble TGF-β1 receptors, and blockade of TGF-β1 activation by decorin (11;21–24). Recently, the use of a small molecule inhibitor of the specific TGF-β receptor ALK5 proved to be effective in suppression of renal fibrosis in rat models of puromycin-induced nephritis and obstructive nephropathy (25;26). In addition, a novel small molecule inhibitor selective for ALK5 and the TGF-β type II receptor was capable of reducing renal fibrosis in diabetic *db/db* mice (27).

Despite the impressive results obtained in experimental renal fibrosis, TGF-β1 blockade has not yet translated into an effective and safe therapeutic in human patients. This might relate to concern about possible adverse effects of TGF-β1 inhibition. The observation that TGF-β1 knockout mice display an autoimmune and hyperinflammatory phenotype, while transgenic mice overexpressing TGF-β1 are protected from renal inflammation, underscores the important role of TGF-β1 in controlling accurate levels of inflammatory activity (28;29). Furthermore, studies in which inhibition of TGF-β1 activity resulted in enhanced tumorigenesis, both in TGF-$\beta1^{+/-}$ mice and in nude mice injected with a dominant-negative mutant TGF-β type II receptor, confirms that TGF-β1 also has relevant functions in anti-proliferation and tumor suppression (30,31).

Thus, considering the multifunctional biological activities of TGF-β1, it is important to explore therapeutic strategies that are aimed at targeting the downstream effectors or

the signaling pathway of TGF-β1 that specifically mediates its fibrogenic action. In this respect, BMP-7 and CTGF have both appeared as promising targets for modulation of profibrotic TGF-β1 activity.

BMP-7 is Downregulated in Renal Fibrosis

Bone morphogenetic protein-7 (BMP-7; OP-1)

The TGF-β superfamily comprises over twenty BMPs, of which BMP-7 (also called osteogenic protein-1 or OP-1) is the most prominent member involved in renal development and disease. BMPs are differentially expressed throughout development and it was demonstrated that in particular BMP-7 and BMP-4 are important in the developing kidney. Initially, BMP-7 is expressed in the ureteric bud. Later in development, BMP-7 is also found in the metanephric mesenchyme, early tubules, and eventually in the podocytes of mature glomeruli (32–35). The relevance of BMP-7 in renal development was observed in homozygous BMP-7 knockout mice, which showed reduction in branching of the ureteric bud and loss of metanephric mesenchyme, resulting in severe renal hypoplasia (32). In the adult kidney, BMP-7 is expressed in glomerular podocytes, the thick ascending limb, the distal convoluted tubule, and most strongly in the collecting duct (36).

BMP-7 expression in renal fibrosis

In contrast to the consistent upregulation of TGF-β1 in models of experimental and human renal fibrosis, expression of BMP-7 was shown to be markedly reduced in experimental diseases associated with renal fibrosis. For example, the expression of BMP-7 mRNA and protein was downregulated after ischemia-reperfusion injury, unilateral ureteral obstruction (UUO), pyelonephritis, chronic allograft nephropathy, and in genetic murine models of Alport syndrome and lupus nephritis (37–43). In streptozotocin-induced diabetic rats, renal expression of BMP-7 was decreased by more than 90%, and this was accompanied by downregulation of the BMP type II receptor and the type I receptor ALK2 (44). Decrease of BMP-7 expression was also evidenced in mouse podocytes cultured under high glucose, and in renal biopsies of patients with diabetic nephropathy (45;46). Thus far, the latter study is the only report available demonstrating decrease of BMP-7 expression in human patients. In addition, loss of BMP-7 signaling activity, as illustrated by lower phosphorylated Smad1/5 protein levels, was observed in experimental nephrotoxic serum nephritis and diabetic nephropathy (47;48).

Efficacy of BMP-7 as an Antifibrotic Drug

***In vitro* antifibrotic effects of BMP-7 in renal cells**

The function of BMP-7 in the adult kidney has not been revealed completely, but recent evidence suggest that endogenous BMP-7 might function as a regulator of kidney homeostasis and regeneration by maintaining a differentiated epithelial phenotype of tubular epithelial cells (48;49). *In vitro* studies have demonstrated that BMP-7 is a potent antagonist of TGF-β1 mediated effects on renal cells. In particular, BMP-7 proved to be a

potent inhibitor of TGF-β1 induced EMT of proximal tubular epithelial cells (47). Other protective effects of BMP-7 in proximal tubular epithelial cells include inhibition of TGF-β1 production, decrease of proinflammatory genes and chemoattractants, and inhibition of EMT induced by multiple myeloma light chains (36;50;51). Also in mesangial cells and podocytes, BMP-7 was able to inhibit adverse effects caused by TGFβ1, aldosterone, or high glucose (45;46;52;53). The remarkable potential of BMP-7 even to reverse fibrosis was demonstrated by its ability to induce formation of epithelial cell aggregates in cultures of adult renal fibroblasts, which was accompanied by acquisition of E-cadherin expression and decreased motility, indicating that true mesenchymal-to-epithelial transition (MET) might also be achieved (54).

BMP-7 therapy in experimental fibrosis

In vivo evidence for antifibrotic properties of BMP-7 was first demonstrated by Vukicevic *et al.* (55), who showed that BMP-7 reduced severity of renal injury after ischemia-reperfusion by temporary bilateral renal artery occlusion in rats. In this study, intravenously administered BMP-7 preserved kidney function as evidenced by reduced blood urea nitrogen and serum creatinine, and it also attenuated proteinuria while increasing survival rate. In subsequent studies, others showed that BMP-7 prevented renal fibrogenesis associated with ureteral obstruction, and effectively restored renal function if administered during the progression of this fibrotic disease (39;56).

The *in vitro* potential of BMP-7 to inhibit EMT and to induce MET was confirmed in a mouse model of chronic injury caused by nephrotoxic serum nephritis, in which reversal of renal fibrosis was observed even with BMP-7 therapy starting several weeks after the occurrence of damage (47;54). The same investigators also showed that BMP-7 therapy could attenuate progression of renal disease in genetic mouse models of lupus nephritis and Alport syndrome (43). However, the therapeutic potential of BMP-7 has been investigated most extensively in experimental diabetic nephropathy, which in human patients is the leading cause of end-stage renal disease. In streptozotocin-induced diabetic rats, both glomerular and tubulointerstitial damage as well as albuminuria were significantly attenuated by BMP-7 therapy in a dose-dependent manner (57). A similar effect on structural changes was observed after BMP-7 treatment in diabetic CD1 mice. In that study, BMP-7 had no effect on albuminuria (58). However, in diabetic transgenic mice overexpressing BMP-7 in podocytes and proximal tubuli, the severity of glomerulosclerosis, interstitial fibrosis, and albuminuria were all markedly decreased (48).

The therapeutic potential of BMP-7 reaches beyond its efficacy in the kidney. In the liver, BMP-7 treatment attenuated CCl4-induced fibrosis by inhibition of hepatocyte-to-fibroblast transition, and it facilitated hepatocyte regeneration, and accelerated restoration of liver function in mice after partial hepatectomy (59;60). However, the antifibrotic effect of BMP-7 in the liver remains controversial, since one study has recently demonstrated that BMP-7 expression was upregulated in the cirrhotic human liver, and that BMP-7 could induce production of collagen and fibronectin in hepatic stellate cells (61). In rats with inflammatory bowel disease, systemic administration of BMP-7 led to less severe colitis with preserved histology and suppression of proinflammatory and profibrogenic genes (62). Administration of BMP-7 also inhibited the progression of cardiac fibrosis in mouse

models of pressure overload and chronic allograft rejection, probably by counteracting TGF-β1 induced endothelial-to-mesenchymal transition (63). In line with its strong osteogenic properties, intraperitoneal injection of BMP-7 was successful in treatment of renal osteodystrophy and adynamic bone disorder in mice (64;65). Paradoxically, however, BMP-7 inhibited vascular calcification in a murine model of renal failure associated atherosclerosis, rendering it almost a panacea (66).
Local BMP-7 therapy is currently under study in human orthopedic patients to improve healing of fractures and non-unions, and for spinal fusion (67). Thus far, clinical trials with BMP-7 have not been performed in renal diseases, and it remains to be established whether ectopic bone formation or other adverse effects might occur after systemic administration of BMP-7.

Possible role for other BMPs

Most of the antifibrotic potential of BMP-7 has been attributed to its ability to antagonize the injurious effects driven by TGF-β1. However, little is known about the mechanism by which the interaction between these two growth factors occurs. Interestingly, the protective effect of BMP-7 on preservation of TGFβ1-induced downregulation of E-cadherin expression in tubular epithelial cells could be mimicked by transfection with the constitutively active general BMP type I receptor ALK3 (47). Furthermore, expression of thrombospondin-1 (TSP-1), which is the major activator of latent TGF-β1 in experimental glomerulonephritis and diabetic nephropathy, was repressed by the prototypical BMP target gene Id1 (68;69). These data suggest that these antifibrotic effects are not entirely specific for BMP-7, and that other BMPs might have similar anti-TGFβ1 activity. In this respect, it is noteworthy that both BMP-4 and BMP-6 were able to functionally substitute for loss of BMP-7 during kidney development (70), and that BMP-6 could inhibit TGFβ1-induced expression of CTGF and plasminogen activator inhibitor-1 in renal interstitial fibroblasts (71). Our observations that myofibroblast progenitor cells derived from patients with diabetes were deficient in BMP-6 expression (71), and that in renal cortex of diabetic mice, BMP-4, -5, and -6 were decreased to a similar extent as BMP-7 (Figure 1), indicate that, in addition to BMP-7, also other BMPs might play an important role as antagonists of renal fibrosis.

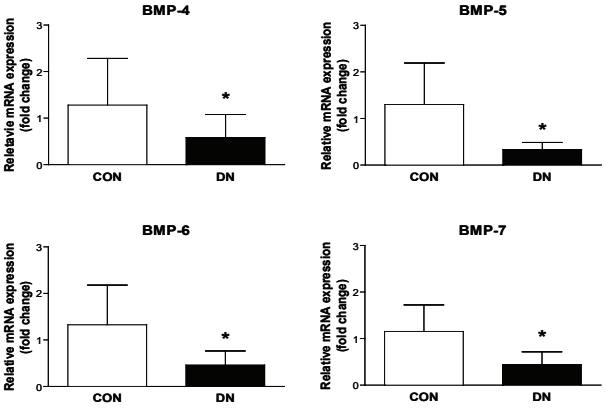

Figure 1. BMP-4, -5, -6, and -7 are similarly decreased in renal cortex of diabetic mice. Diabetic nephropathy (DN) was induced in 12-week old female C57Bl6/J mice by intraperitoneal injection with streptozotocin (n=16). Renal cortex was harvested 9 weeks after induction. Expression of BMP genes was assessed by quantitative PCR. Results are expressed as mean±SD (*P<0.05).

Despite apparent overlap in functions of other BMPs with BMP-7, it should be kept in mind that distinct biological effects have been attributed to different BMPs. For instance, BMP-6 and BMP-7 exhibited differential and specific effects on survival and outgrowth of neurons, and opposite effects of BMP-2 and BMP-7 have been reported on renal branching morphogenesis (72;73). Moreover, the inhibitory effect of BMP-7 on vascular calcification was not observed with BMP-2 and BMP-4, which were both shown to promote calcification of vascular smooth muscle cells (74;75).

Modulators of BMP-7

The activity of BMP-7 in the kidney can be enhanced or inhibited by extracellular modulators. Recently, kielin/chordin-like protein (KCP) was identified as a novel enhancer of BMP signaling. $KCP^{-/-}$ mice, which have less pSmad1 protein, developed significantly more renal damage and interstitial fibrosis after induction of UUO or acute tubular necrosis (76). In addition, pSmad2 protein levels were increased in $KCP^{-/-}$ mice, suggesting that the antifibrotic effect of KCP also included inhibition of TGF-β1 activity (77). Also, several extracellular modulators have been identified that inhibit BMP-7 activity, including uterine sensitization-associated gene (USAG)-1, which is abundantly expressed in the kidney. USAG-$1^{-/-}$ mice had increased pSmad1/5 protein levels and exhibited prolonged survival and preserved renal function after cisplatin nephrotoxicity or UUO. The renoprotection in these mice could be abolished by administration of a neutralizing antibody against BMP-7 (78). Moreover, in mice with folic acid nephrotoxicity, the renal expression of USAG-1 at day 7 correlated with serum creatinine at day 14, suggesting that USAG-1 expression in a kidney biopsy could be useful in predicting outcome (79). Recently, SclerOSTin domain-containing-1 or SOSTDC1 was identified as the human ortholog of USAG-1 and was shown to be highly expressed in normal kidney, but downregulated in renal clear cell carcinoma (80). Other antagonists of BMP-7 in the kidney include gremlin and noggin, of which overexpression of the latter in podocytes has been shown to result in massive mesangial matrix expansion (81;82).

Taken together, these data indicate that the antifibrotic BMP activity in the kidney is not determined by the expression levels of BMP-7 alone, but that expression of multiple other BMPs and BMP modulators might be equally important. Moreover, the previously cited efficacy of inhibiting TGF-β1 without BMP suppletion indicates that, more in general, modulation of the balance between BMP- and TGF-β1-signaling activity might be the key to restoration of appropriate response to injury and reversal of fibrosis. In this respect, a modulator of particular interest is CTGF, which is able to both enhance TGF-β1 activity and inhibit BMP-4 activity (83).

CTGF is an Extracellular Modulator of Multiple Signaling Activities

Connective tissue growth factor (CTGF; CCN-2)

CTGF was first identified in conditioned media of endothelial cells as a 36–38 kDa cysteine-rich polypeptide containing chemotactic and mitogenic activity towards fibroblasts (84). Subsequently, CTGF was acknowledged as a member of the CCN (CTGF/Cyr61/Nov) family, and became also known as CCN-2 (85). The CCN family consists of

six matricellular regulatory proteins, consistent with the notion that they act by binding to extracellular signaling molecules via their multiple distinct interaction domains, rather than by signaling through direct binding to unique receptors specific to individual CCNs (86).

Domain 1 of CTGF consists of an N-terminal insulin-like growth factor binding protein, which is involved in binding of CTGF to insulin-like growth factor (IGF)-1 and fibronectin (87;88). Domain 2 of CTGF shares high homology with Von Willebrand Factor type C repeats as well as with chordin, and is responsible for binding of CTGF to TGF-β1 and BMP-4 (83). Domain 3 of CTGF contains a TSP-1 repeat and is involved in binding to vascular endothelial growth factor (VEGF), as well as binding to low-density lipoprotein receptor-related protein (LRP)-1 and integrin α6β1 (89–91). Domain 4 of CTGF is a C-terminal cysteine-rich domain. This domain is involved in binding of CTGF to heparan sulfate proteoglycans (HSPG), integrin αvβ3, fibronectin, and to the Wnt co-receptor LRP-6 (88;92–94). Domain 4 is presumed to also interact with the neurotrophin receptor TrkA, which leads to induction of the transcription factor TGF-β-inducible early gene (TIEG) (95). In addition, CTGF contains a cysteine-free hinge region between domain 2 and 3, which is susceptible to proteolytic cleavage by matrix metalloproteinases (MMP) and other proteases (96).

Functions of CTGF

The biological functions of CTGF are complex and diverse. Importantly, CTGF is required for most of the increased ECM production and other profibrotic activity generally observed in response to TGF-β1 (97). In addition, CTGF is critically involved in cell growth and differentiation, migration, adherence, apoptosis and survival, as well as in angiogenesis and chondrogenesis (98). Specific direct effects of CTGF on renal cells include migration, hypertrophy, fibronectin production, and actin disassembly in mesangial cells, EMT and fibronectin production of tubular epithelial cells, and collagen type III and TSP-1 production by renal interstitial fibroblasts (99–104). Recently, a physiological role for CTGF has been demonstrated in human corneal epithelial cells, in which CTGF was required for regulation of re-epithelialization (105).

Given its many and distinct interaction domains, it seems likely that CTGF functions mainly as an extracellular modulator of signaling activity of other growth factors. For instance, upon binding of domain 1 to IGF-1, CTGF and IGF-1 act synergistically to induce collagen type I and type III production by renal interstitial fibroblasts (106). In contrast, binding of CTGF to BMP-4 results in inhibition of BMP-4 signaling activity (83). Binding of CTGF to VEGF via domain 3 results in inhibition of VEGF-induced angiogenesis, and this binding and inhibition are both lost upon cleavage of the CTGF hinge region by MMP-1, -3, and -13 (96). Binding of CTGF domain 3 to LRP-1 results in activation of ERK1/2 MAPK signaling, which can be inhibited by the LRP-1 antagonist receptor-associated protein (107). Furthermore, simultaneous interaction of CTGF with HSPG and integrins is essential for its role in adherence and migration, and binding of CTGF domain 4 to LRP-6 suppresses Wnt-signaling (92;108). Also, binding of CTGF to fibronectin via domain 1 and domain 4 is important for the function of CTGF in cell-matrix interactions (88;94).

Synergy between CTGF and TGF-β1

CTGF is not only induced by TGF-β1, but it is also a major enhancer of the biological activity of TGF-β1. For example, persistence of skin fibrosis in newborn mice was achieved only after co-injection of both TGF-β1 and CTGF, and not after injection of TGF-β1 or CTGF alone (109). Anchorage-independent growth of rat fibroblasts and collagen synthesis were also dependent on both TGF-β1 and CTGF (110;111).

The synergy between CTGF and TGF-β1 might be explained by several mechanisms (Figure 2). In the first place, physical interaction between TGF-β1 and domain 2 of CTGF enhanced receptor binding and potentiated Smad-signaling activity of TGF-β1 (83). In addition, binding to TrkA and induction of TIEG, presumably by domain 4 of CTGF, might interrupt the negative feedback loop of TGF-β1, since Smad7, the major inhibitory Smad of the TGF-β1 signaling pathway, is suppressed by TIEG (95). Finally, binding of CTGF domain 3 to LRP-1 enhanced TGF-β1 responses, including myofibroblast activation, *de novo* expression of α-smooth muscle actin, and extracellular accumulation of fibronectin. Remarkably, the latter effects were mediated by ERK1/2 MAPK activation, without any evident modulation of TGF-β1 induced phosphorylation of Smad2, or of pSmad2/3 association with Smad4 (107).

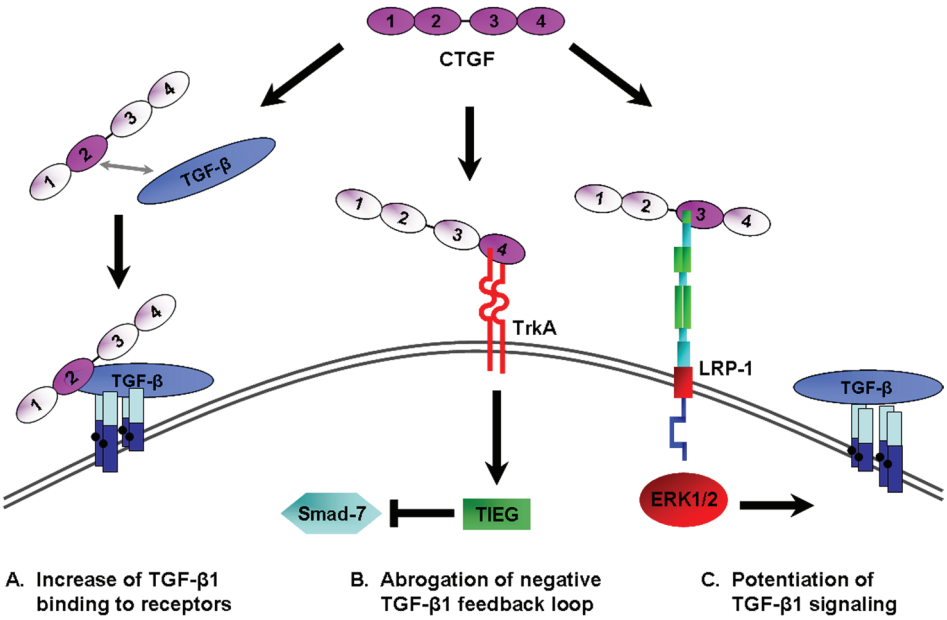

Figure 2. Mechanisms behind the synergy between CTGF and TGF-β1. A: Direct binding of TGF-β1 to domain 2 of CTGF enhances receptor binding and potentiates signaling activity of TGF-β1 (83). B Direct binding of CTGF, presumably via domain 4, to TrkA leads to induction of TIEG with subsequent inhibition of Smad7, the major inhibitory Smad of the TGF-β1 signaling pathway (95). Loss of inhibitory Smad7 activity would lead to enhanced TGF-β1 signaling activity. C Binding of CTGF domain 3 to LRP-1 enhances TGF-β1 responses by ERK1/2 MAPK activation (107).

It remains to be established whether synergy between TGF-β1, TrkA, and LRP-1 derives entirely from their individual interactions with separate CTGF domains *per se*. In addition, cross-talk resulting from simultaneous interaction of the distinct CTGF domains with their respective binding partners might be relevant. This would be of interest with respect to biological relevance of full length CTGF, as compared to physiologically occurring CTGF cleavage products.

CTGF is Upregulated in Renal Fibrosis

CTGF expression in renal fibrosis

Although development of the kidney is not affected in $CTGF^{-/-}$ mice (112), the expression of CTGF is more abundant in the normal adult kidney than in other organs (113). In control human renal biopsies, CTGF mRNA is mainly expressed in podocytes, and in some parietal epithelial cells and interstitial cells. However, in renal biopsies of patients with progressive glomerulopathies and tubulointerstitial damage, strong overexpression of glomerular and tubulointerstitial CTGF mRNA was reported, of which the latter correlated with the degree of damage (114). In experimental and human crescentic glomerulonephritis, CTGF was strongly overexpressed in areas with proliferating glomerular parietal epithelial cells (115;116). Upregulation of CTGF was also observed in experimental models of lupus nephritis, anti-Thy-1.1-induced acute mesangial proliferative glomerulonephritis, UUO, hypertensive nephrosclerosis, and in the remnant kidney of 5/6 nephrectomized mice (117–121). Furthermore, CTGF is critically involved in chronic allograft nephropathy, in which CTGF expression was shown to be highly expressed in tubular epithelial cells of the transplanted kidney, and in serum and urine of renal transplant recipients (122;123).

CTGF in diabetic nephropathy

CTGF is considered to be of particular interest to diabetic nephropathy. Already soon after its identification, the expression of CTGF mRNA was shown to be strongly upregulated in human mesangial cells cultured under high glucose, and in renal biopsies of patients with diabetic nephropathy (114;124). These observations were extended in rodent models of both type 1 and type 2 diabetic nephropathy (104;125;126). Furthermore, severity of diabetic nephropathy was aggravated in transgenic mice with specific overexpression of CTGF in podocytes (127). Upregulation of CTGF was also confirmed in the diabetic myocardium, liver, retina, and aorta (126;128–130).

The potential use of CTGF as a biomarker for renal damage in diabetes was assessed in several clinical studies, in which soluble levels of CTGF were increased in plasma and urine of patients with diabetic nephropathy, and correlated with clinical markers of renal disease (131–134). Moreover, in renal biopsies of patients with type 2 diabetic nephropathy, tubular expression of CTGF correlated with proteinuria, serum creatinine, and interstitial fibrosis (135). Interestingly, CTGF expression was shown to be decreased in microdissected glomeruli from renal biopsies of patients with diabetic nephropathy, but this was probably related to loss of podocytes (136).

Regulation of CTGF

TGF-β1 is the earliest recognized inducer of CTGF, and has remained one of the most studied regulators of CTGF expression in fibrotic processes (97;137). In addition, *in vitro* studies with renal cells demonstrated that CTGF is also a direct target of gene regulation by TGF-β1-independent factors including high glucose, angiotensin II, aldosterone, hypoxia inducible factor-1α, and cyclic mechanical strain (124;125;138–141). The 3'-untranslated region of CTGF gene also contains regulatory sequences, of which the activity depends on as yet largely unidentified factors (142;143). In addition, several polymorphisms in the CTGF promoter region have been identified, which might contribute to genetic susceptibility of patients to develop fibrotic disorders (144–146). Of great interest, a G-945C polymorphism in the promoter of CTGF was recently shown to be associated with susceptibility to systemic sclerosis (147). However, it remains to be established whether this polymorphism is also associated with increased levels of CTGF and susceptibility of patients to develop renal fibrosis.

CTGF is a Target for Antifibrotic Therapy

Inhibition of CTGF

CTGF is a downstream mediator of profibrotic TGF-β1 activity, and although one report has proposed a role of CTGF in mediating TGF-β1 induced apoptosis in a breast cancer cell line (148), there are no other reports of CTGF involvement in the beneficial functions of TGF-β1 in anti-inflammation and tumor suppression. In addition, restoration of the balance between TGF-β1 and BMP signaling activity seems more attractive than interference in a pathway that also includes beneficial effects. Therefore, targeting CTGF in fibrotic disorders, and renal fibrosis in particular, has been proposed as a more suitable approach for antifibrotic intervention than direct targeting of TGF-β1 (149;150).
In vitro, EMT of tubular epithelial cells could be inhibited both by a homologous hexadeca-peptide of domain 4 of CTGF that recognizes αvβ3, and by CTGF knockdown with antisense oligodeoxynucleotides (ODN) (151;152). CTGF antisense ODN treatment also effectively reduced expression of ECM genes and tubulointerstitial fibrosis in experimental renal diseases, including UUO, the remnant kidney model in TGF-β1 overexpressing mice, and in dexamethasone-induced nephropathy (153–155).

CTGF therapy in diabetic nephropathy

Recently, treatment with CTGF antisense ODN proved to be successful in attenuation of proteinuria, and in reduction of genes involved in mesangial matrix expansion in mouse models of type 1 and type 2 diabetes (156). Interestingly, reduction of albuminuria was also observed in diabetic *db/db* mice treated with CTGF neutralizing antibody, and in diabetic $CTGF^{+/-}$ mice harboring only one functional CTGF allele. These latter two studies also showed that inhibition of CTGF in diabetic nephropathy resulted in reduced thickening of the glomerular basement membrane (Flyvbjerg *et al.*, abstract J Am Soc Nephrol 15:261A, 2004, and Nguyen *et al.*, submitted). Anti-CTGF treatment also reversed vascular stiffness and microvascular leakage, improved cardiac function, and normalized hypertension in type 1 diabetic rats (Langsetmo *et al.*, abstract Diabetes

55:A122, 2006). Moreover, a fully human anti-CTGF antibody (FG3019) has been tested for safety in phase 1 clinical trials in microalbuminuric type 1 and type 2 diabetic patients. Administration of the antibody proved to be well tolerated and was able to decrease urinary albumin to creatinine ratio by 50% or more in 7 of 19 subjects (Adler *et al.*, abstract J Am Soc Nephrol 15:TH-PO239, 2004).

Conclusion

Renal fibrosis is the major determinant in progression of acute and chronic kidney disease. TGF-β1 is the driving force behind renal fibrosis and has since long been regarded as the key factor to be targeted in prevention and treatment of renal fibrosis. Considering its multiple effects, which are in part also protective and beneficial, direct inhibition of general TGF-β1 activity might not be clinically applicable.
Recently, BMP-7 and CTGF have emerged as novel modulators of profibrotic TGF-β1 activity. In experimental models of renal disease, especially in diabetic nephropathy, either administration of BMP-7 or inhibition of CTGF have been sufficient to result in striking improvement of renal function and structure. Targeting the profibrotic activity of both growth factors might occur at several levels of their pathophysiological interaction with other mediators, extracellular matrix, and cell surface molecules. However, for both candidate therapeutics, assessment of clinical applicability is still in an early phase of testing.

Acknowledgments

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Chapter IX

Summary and Perspectives

Summary

Diabetes mellitus is characterized by inappropriately high blood glucose levels resulting from low levels of insulin and/or from abnormal resistance to the effects of insulin. Patients with diabetes have an increased risk to develop severe complications, of which diabetic nephropathy is the main focus in this thesis. Currently, diabetic nephropathy is the most important cause of end-stage renal disease (ESRD) in large parts of the world and contributes significantly to mortality in renal patients.

Chapter I is an introduction to the field of growth factor involvement in diabetic nephropathy, and describes the current pathogenic concepts of, and risk factors for this disease. Diabetic nephropathy is characterized by proteinuria and accumulation of extracellular matrix (ECM) in the kidney. The natural course of diabetic nephropathy is unpredictable and the pathogenesis of progression is not completely understood, despite expanding data from experimental and clinical studies. Thus, it is important to improve the identification and management of patients with diabetic nephropathy. In this respect, connective tissue growth factor (CTGF) and bone morphogenetic proteins (BMPs) have recently emerged as promising targets.
CTGF is a key factor in ECM production and other profibrotic activity mediated by transforming growth factor (TGF)-β1. CTGF has been associated with numerous fibrotic disorders, but is of special interest to diabetic nephropathy. The expression of CTGF is strongly upregulated in cells cultured under high glucose, and in the diabetic kidney *in vivo*. Furthermore, CTGF is increased in plasma and urine of patients with diabetic nephropathy, and inhibition of CTGF successfully attenuated functional and structural damage in experimental diabetic nephropathy.
BMPs are growth factors that belong to the TGF-β superfamily. BMPs are mainly involved in regulation of biological processes during development. From over twenty different BMPs, only the role of BMP-7 has been described in relation to diabetic nephropathy. The expression of BMP-7 is downregulated in rodent models of diabetic nephropathy, and in renal biopsies of patients with diabetic nephropathy. Moreover, administration of BMP-7 attenuated both glomerular and tubulointerstitial damage as well as albuminuria in diabetic rats.

Chapter II reports urinary excretion of CTGF (U-CTGF) in a large cross-sectional study of 318 patients with type 1 diabetes and 20 normoglycemic control subjects. U-CTGF was significantly higher in patients with diabetic nephropathy than in microalbuminuric and normoalbuminuric patients, and in control subjects. In patients with diabetic nephropathy, U-CTGF correlated with urinary albumin excretion (UAE) and inversely with glomerular filtration rate (GFR), both important clinical markers for severity of renal disease. Furthermore, a standardized (1-SD) increase of U-CTGF resulted in 2.3-fold increased chance of diabetic nephropathy, which was comparable with the odds ratios for diabetic nephropathy of hypertension and hyperglycemia. The observations in this study indicate that U-CTGF might be regarded as a novel risk factor for development of diabetic nephropathy.

Chapter III addresses the predictive value of baseline plasma CTGF for outcome and disease progression in a prospective study of patients with type 1 diabetic nephropathy. Plasma CTGF was significantly increased in 198 patients with diabetic nephropathy as compared to 188 patients with normoalbuminuria. As in the previous study, a standardized (1-SD) increase of plasma CTGF resulted in 2.0-fold increased chance of diabetic nephropathy, and plasma CTGF correlated with UAE and inversely with GFR. Plasma CTGF at baseline was associated with a higher rate of decline in GFR. Thus far, the only parameter known to be associated with decline in renal function of patients with diabetic nephropathy had been baseline levels of albuminuria. Our study revealed that plasma CTGF significantly contributes to this correlation. Baseline plasma CTGF was also an independent predictor of ESRD and mortality in patients with diabetic nephropathy. Moreover, in diabetic patients with nephrotic range albuminuria, plasma CTGF was the only parameter predicting ESRD. These observations suggest that CTGF might be an important pathogenic factor in progression of diabetic nephropathy, and that plasma CTGF might find clinical application as a biomarker.

Chapter IV describes the prediction of baseline U-CTGF for deterioration of renal function in patients with idiopathic membranous nephropathy (iMN), which is one of the most frequent primary glomerular diseases in non-diabetic patients. Baseline U-CTGF was measured in 51 patients with nephrotic iMN and normal renal function at presentation. Baseline urinary CTGF excretion was significantly higher in iMN patients who reached renal death. This illustrates that CTGF might be a useful biomarker also in patients with non-diabetic renal disease. Another striking observation was the strong correlation between U-CTGF and urinary β-2-microglobulin excretion. This suggests that filtered CTGF is reabsorbed from the primary urine by proximal tubular epithelial cells, and that increased U-CTGF in patients with iMN reflects tubular dysfunction.

Chapter V deals with the possible interplay between CTGF and BMP-7 in diabetic nephropathy. For this, diabetes was induced in transgenic $CTGF^{+/-}$ mice, which harbor only one functional CTGF allele. The approximately 50% lower CTGF mRNA and protein in diabetic $CTGF^{+/-}$ mice was associated with absence of glomerular basement membrane (GBM) thickening and preserved matrix metalloproteinase (MMP)-activity, and with less severe albuminuria as compared to diabetic wild-type $CTGF^{+/+}$ mice. Although renal BMP-7 mRNA was similar in diabetic $CTGF^{+/+}$ and $CTGF^{+/-}$ mice, phosphorylation of the BMP-signal transduction protein Smad1/5 and expression of the BMP-target gene Id1 were more preserved in diabetic $CTGF^{+/-}$ mice. Moreover, renal Id1 mRNA expression correlated with albuminuria and MMP-activity. In normoglycemic mice, a decrease of pSmad1/5 in renal cortex was observed after intraperitoneal injection of CTGF. Also in cultured renal cells, co-stimulation of BMP-7 with CTGF diminished BMP-7 signaling activity. Furthermore, CTGF was able to bind BMP-7 with high affinity. This study demonstrates that CTGF has a pathogenic role in major aspects of diabetic nephropathy and that CTGF is able to bind directly to BMP-7 and act as an inhibitor of BMP signaling activity, both *in vivo* and *in vitro*.

Chapter VI addresses the relation between outgrowth of myofibroblast progenitor cells (MFPC) and aberrant expression of BMP family members in patients with type 1 diabetes. Blood from six patients with type 1 diabetes yielded significantly higher numbers of MFPC than blood from six matched control subjects. This involved increased proliferation and decreased apoptosis. Targeted screening by microarray analysis showed that BMP-6 expression was downregulated in MFPC derived from patients with diabetes. Furthermore, an inverse correlation was observed between BMP-6 mRNA level and the number of MFPC. In renal interstitial fibroblasts, BMP-6 antagonized TGF-β1 induced gene expression of plasminogen activator inhibitor-1 and CTGF. These data suggest that increase of BMP-6 deficient MFPC in patients with diabetes probably occurs via robust alternative programming. Considering the relevance of BMP-6 in angiogenesis and its anti-TGF-β effects, both the excess numbers of MFPC and their lack of BMP-6 secretion may favor adverse tissue remodeling in patients with diabetes.

Chapter VII reports the impact of BMP-6 deficiency on the number of circulating MFPC and on the course of renal fibrosis in a mouse model of chronic obstructive nephropathy. For this, unilateral ureteral obstruction (UUO) was induced in transgenic BMP-6 null mice, in which exon 2 of the BMP-6 gene was deleted. Compared to wild-type mice, BMP-6 null mice had higher numbers of circulating MFPC. BMP-6 mRNA was upregulated in the obstructed kidneys of wild-type mice, whereas BMP-7 mRNA was similarly decreased in the obstructed kidney of wild-type mice and BMP-6 null mice. In the obstructed kidney of BMP-6 null mice, BMP target gene expression was less increased, whereas αSMA mRNA was significantly higher than in wild-type mice. The number of infiltrating MFPC was very limited in both groups. These data suggest that the number of circulating MFPC is determined by endogenous BMP-6 expression and that loss of BMP-6 results in more severe renal fibrosis.

Chapter VIII reviews the current knowledge of BMP-7 and CTGF in the kidney, and discusses their therapeutic potential in renal fibrosis. Renal fibrosis is the major determinant in progression of kidney disease and results from an inappropriate response to acute and chronic kidney injury. TGF-β1 is the driving force behind renal fibrosis and has since long been regarded as the key factor to be targeted in prevention and treatment of renal fibrosis. Despite the impressive results obtained in experimental renal fibrosis, TGF-β1 blockade has not yet translated into an effective and safe therapeutic in human patients. Therefore, it remains important to explore the role of additional growth factors which are involved in renal regeneration and fibrosis. In experimental models of renal disease, especially in diabetic nephropathy, either administration of BMP-7 or inhibition of CTGF have been sufficient to result in striking improvement of renal function and structure. Targeting the profibrotic activity of both growth factors might occur at several levels of their pathophysiological interaction with other mediators, extracellular matrix, and cell surface molecules. However, for both candidate therapeutics, assessment of clinical applicability is still in an early phase of testing.

Perspectives

The studies in this thesis demonstrate that CTGF is a pathogenic factor in diabetic nephropathy and that measurement of soluble CTGF levels in patients with diabetic nephropathy might be helpful to identify those patients that are at risk for rapid deterioration of renal function. At present, clinical application of such a test seems not to be of additive value, because plasma CTGF is not a directly modifiable risk factor and because, given the current therapeutic options, these patients already receive optimal treatment. Nevertheless, progression to end-stage renal disease is often inevitable. However, measuring plasma CTGF might in the future contribute to monitoring of efficacy of treatment strategies that affect CTGF levels also indirectly.

Our preclinical studies clearly indicate that CTGF has a pathogenic role in major aspects of diabetic nephropathy. Previously, inhibition of TGF-β and VEGF signaling, or administration of BMP-7 has resulted in comparable decrease of structural and functional renal damage in experimental models of diabetic nephropathy. Considering the role of CTGF as a modulator of these growth factors, as revealed in part by the work described in this thesis, these findings suggest that anti-CTGF therapy might be an attractive strategy for future intervention modalities.

A range of antibodies has been generated which are directed against each of the four functional domains of the CTGF molecule. It remains to be established whether these antibodies have different profiles in terms of *in vitro* and *in vivo* modulation of CTGF bioactivity. Interestingly, a fully human anti-CTGF antibody binding domain 2 of CTGF has been shown to inhibit renal and cardiovascular damage in experimental diabetes. Although domain 2 is predicted to be involved in modulation of TGF-β and BMP signaling, the mechanism behind the therapeutic efficacy of this antibody has not been fully elucidated. It will be exciting to compare pharmaceutical profiles of this and other modalities of anti-CTGF therapy in future comparative preclinical studies, especially in the context of recently initiated phase 1b clinical trials with the anti-CTGF domain 2 antibody (FG3019) in diabetic nephropathy and other fibrotic disorders.

Epilogue

In this thesis, I have shown that disturbance of the balance between CTGF and BMPs is a major determinant of excessive fibrosis, in particular in diabetic nephropathy. Limited scar formation in reaction to injury can be beneficial, but optimal regeneration will require balance between profibrotic and antifibrotic factors. For me, this is the *yin* and *yang* of renal response to injury.

Chapter X

Nederlandse Samenvatting

Samenvatting

Diabetes mellitus wordt gekenmerkt door te hoge bloedglucose spiegels. Dit kan een gevolg zijn van verminderde productie van insuline en/of resistentie van het lichaam voor de effecten van insuline. Patiënten met diabetes hebben een verhoogd risico op het ontwikkelen van ernstige complicaties, waarvan diabetische nefropathie het focus is van dit proefschrift. Momenteel is diabetische nefropathie in grote delen van de wereld de belangrijkste oorzaak van eindstadium nierfalen (ESRD) en draagt het in grote mate bij aan verlies van kwaliteit van leven en aan mortaliteit van patiënten met diabetes.

Hoofdstuk I is een introductie in het veld van groeifactoren die betrokken zijn bij diabetische nefropathie. Diabetische nefropathie wordt gekenmerkt door albuminurie en accumulatie van extracellulaire matrix (ECM) in de nier. Ondanks veel data verkregen uit experimentele en klinische studies, blijft het natuurlijke beloop van diabetische nefropathie onvoorspelbaar en is de pathogenese van ziekteprogressie nog niet geheel opgehelderd. Het is belangrijk de identificatie en de behandeling van patiënten met diabetische nefropathie te verbeteren. Hiervoor lijken de groeifactoren connective tissue growth factor (CTGF) en bone morphogenetic proteins (BMPs) geschikte opties te bieden.
CTGF is een factor die betrokken is bij ECM productie en bij andere profibrotische effecten van transforming growth factor (TGF)-β1, de meest belangrijke stimulans van littekenvorming. CTGF is in verband gebracht met talloze fibrotische aandoeningen, maar in het bijzonder met diabetische nefropathie. De expressie van CTGF is sterk toegenomen in niercellen die gekweekt zijn in aanwezigheid van hoge glucose concentraties en in de diabetische nier. Daarnaast zijn CTGF spiegels verhoogd in plasma en urine van patiënten met diabetische nefropathie en leidt remming van CTGF tot minder functionele en structurele schade in diermodellen van diabetische nefropathie.
BMPs zijn groeifactoren die behoren tot de TGF-β superfamilie. BMPs zijn vooral betrokken bij de regulatie van biologische processen tijdens de ontwikkeling. Van de meer dan twintig verschillende BMPs, is alleen van BMP-7 een rol beschreven in relatie tot diabetische nefropathie. De expressie van BMP-7 is verlaagd in nieren van diabetische muizen en ratten en in nierbiopten van patiënten met diabetische nefropathie. Bovendien leidt toediening van BMP-7 aan diabetische ratten tot sterke vermindering van zowel glomerulaire en tubulointerstitiële nierschade, als van albuminurie.

Hoofdstuk II betreft een studie naar de betekenis van de excretie van CTGF in de urine (U-CTGF) in een cross-sectionele studie van 318 patiënten met type 1 diabetes. U-CTGF bleek significant hoger te zijn in patiënten met diabetische nefropathie dan in microalbuminurische en normoalbuminurische patiënten, en dan in controle personen. U-CTGF correleerde positief met de urine excretie van albumine (UAE) en negatief met de glomerulaire filtratiesnelheid (GFR), beide belangrijke klinische markers voor de ernst van diabetische nierschade. Bovendien bleek een gestandaardiseerde (1-SD) toename van U-CTGF te resulteren in een 2.3-maal verhoogde kans op diabetische nefropathie. Deze kans was vergelijkbaar met de odds ratio's voor diabetische nefropathie van hypertensie en hyperglykemie. De resultaten van deze studie suggereren dat U-CTGF een nieuwe risico-indicator is voor het ontstaan van diabetische nefropathie.

Hoofdstuk III is een studie naar de voorspellende waarde van plasma CTGF voor eindpunten en ziekteprogressie van type 1 diabetische nefropathie. Plasma CTGF bleek significant hoger te zijn in 198 patiënten met diabetische nefropathie dan in 188 patiënten met normoalbuminurie. Net als in de vorige studie gevonden werd voor U-CTGF, resulteerde een 1-SD toename van plasma CTGF in een 2.0-maal verhoogde kans op diabetische nefropathie. Tevens correleerde plasma CTGF positief met UAE en negatief met GFR. De spiegel van plasma CTGF aan het begin van de studie bleek geassocieerd te zijn met de snelheid waarmee de GFR daalde in het verdere beloop van de studie. Tot dusverre was albuminurie de enige indicator voor de snelheid van toekomstige GFR daling. Ook na correctie voor albuminurie, bleek plasma CTGF significant te correleren met snelheid van nierfunctieverlies. Bovendien was CTGF een onafhankelijke voorspeller van ESRD en mortaliteit in patiënten met diabetische nefropathie. De voorspellende waarde was het sterkst in patiënten met nefrotische range albuminurie. In deze patiënten bleek plasma CTGF zelfs de enige parameter te zijn die ESRD kon voorspellen. Deze data suggereren dat CTGF een belangrijke rol speelt in de progressie van diabetische nefropathie en dat plasma CTGF waardevol zou kunnen zijn als klinische biomarker.

Hoofdstuk IV is gewijd aan de voorspellende waarde van U-CTGF voor achteruitgang van nierfunctie in patiënten met idiopathische membraneuze glomerulopathie (iMN), een van de meest voorkomende primaire glomerulaire aandoeningen in niet-diabetische patiënten. U-CTGF werd gemeten in 51 patiënten met nefrotische iMN die bij aanvang van de studie een normale nierfunctie hadden. U-CTGF bleek significant verhoogd te zijn in patiënten met iMN die in de loop van de studie nierfalen ontwikkelden. Dit geeft aan dat CTGF mogelijk ook toegepast kan worden als biomarker in patiënten met niet-diabetische nierziekten. Opmerkelijk was verder dat U-CTGF zeer sterk correleerde met de excretie van β-2-microglobuline in de urine. Dit suggereert dat CTGF uit de primaire urine wordt gereabsorbeerd door proximale tubulusepitheelcellen en dat verhoogde U-CTGF in patiënten met iMN het gevolg is van tubulaire dysfunctie.

Hoofdstuk V behandelt de interactie tussen CTGF en BMP-7 in diabetische nefropathie. Diabetes werd geïnduceerd in transgene $CTGF^{+/-}$ muizen. Deze hebben slechts één functioneel CTGF allel, waardoor de CTGF expressie circa 50% lager was dan in diabetische wild-type $CTGF^{+/+}$ muizen. Dit was geassocieerd met behoud van een normale dikte van de glomerulaire basaalmembraan, behoud van matrix metalloproteinase (MMP) activiteit en minder ernstige albuminurie. Hoewel de genexpressie van BMP-7 in de nier vergelijkbaar was met die in diabetische $CTGF^{+/+}$ muizen, waren de fosforylatie van het BMP signaal transductie eiwit Smad1/5 en de expressie van het BMP target gen Id1 beter gepreserveerd in diabetische $CTGF^{+/-}$ muizen. Bovendien bleek de Id1 expressie in de nier te correleren met albuminurie en MMP activiteit. Het effect van CTGF op BMP signalering werd bevestigd door verlaging van pSmad1/5 in de niercortex na intraperitoneale injectie van CTGF in normoglykemische muizen. Ook in gekweekte niercellen leidde co-stimulatie met BMP-7 en CTGF tot verlaging van de BMP-7 signaleringsactiviteit. Deze remming berust vermoedelijk op de directe binding die wij konden aantonen van CTGF aan BMP-7. Interferentie met BMP signaleringsactiviteit door CTGF blijkt dus een pathogenetische rol te spelen in belangrijke aspecten van diabetische nefropathie.

Hoofdstuk VI betreft onderzoek naar de relatie tussen myofibroblast progenitorcellen (MFPC) en expressie van BMP genen in patiënten met type 1 diabetes. Het aantal MFPC gekweekt uit perifeer bloed van zes patiënten met type 1 diabetes was significant hoger dan van zes controle personen. Dit berustte op zowel een toegenomen proliferatie als een afgenomen apoptotische activiteit. Een microarray analyse identificeerde BMP-6 als een gen waarvan de expressie was verlaagd in MFPC van patiënten met diabetes. Verder bleek de BMP-6 mRNA expressie negatief gecorreleerd te zijn met het aantal MFPC. Bovendien was BMP-6 in staat om de TGF-β1 geïnduceerde genexpressie van PAI-1 en CTGF te antagoneren, zoals eerder aangetoond voor BMP-7. De persistentie van verschillen tussen gekweekte MFPC van patiënten en controle personen, ondanks identieke kweekomstandigheden, suggereert dat deze progenitorcellen *in vivo* robuuste alternatieve programmering hadden ondergaan door het diabetische milieu. Gezien het belang van BMP-6 in angiogenese en de anti-TGF-β effecten van BMP-6, zou in patiënten met diabetes zowel het hoge aantal MFPC als het gebrek aan BMP-6 productie van deze cellen kunnen leiden tot een gestoorde herstelreactie van het weefsel.

Hoofdstuk VII beschrijft de invloed van BMP-6 deficiëntie op het aantal MFPC en op het beloop van nierfibrose in een model van chronische obstructieve nefropathie. Hiervoor werd unilaterale ureterobstructie (UUO) toegepast in transgene BMP-6 knockout muizen, waarin exon 2 van het BMP-6 gen ontbreekt. Vergeleken met wild-type muizen, hadden BMP-6 knockout muizen een hoger aantal circulerende MFPC. BMP-6 mRNA was verhoogd in de geobstrueerde nieren van wild-type muizen, terwijl BMP-7 mRNA even sterk verlaagd was in de geobstrueerde nier van wild-type muizen en van BMP-6 knockout muizen. In de geobstrueerde nier van BMP-6 knockout muizen was de BMP target gen expressie minder toegenomen, terwijl α-SMA sterker was verhoogd dan in wild-type muizen. De genexpressie van MCP-1 en de influx van het aantal macrofagen was gelijk in beide groepen. Het aantal infiltrerende MFPC was slechts zeer beperkt in beide groepen. De resultaten van deze studie suggereren dat het aantal circulerende MFPC wordt bepaald door endogene BMP-6 expressie en dat verlies van BMP-6 leidt tot ernstiger nierfibrose.

Hoofdstuk VIII is een overzicht van bestaande literatuur over de therapeutische mogelijkheden van BMP-7 en CTGF in nierfibrose, aangevuld met enkele originele data. Nierfibrose is de belangrijkste determinant in progressie van nierziekte en ontstaat in reactie op acute en chronische nierschade. TGF-β1 is de drijvende kracht achter nierfibrose en wordt sinds lange tijd beschouwd als het focus voor antifibrotische therapie. Ondanks indrukwekkende resultaten verkregen met TGF-β1 remming in proefdierstudies, heeft dit zich nog niet vertaald in een effectief en veilig therapeuticum dat toegepast kan worden in patiënten. Daarom blijft het belangrijk om de rol van andere groeifactoren te onderzoeken die betrokken zijn in regeneratie en fibrose van de nier. In experimentele modellen van nierziekten, met name ook in diabetische nefropathie, hebben zowel toediening van BMP-7 als remming van CTGF geresulteerd in aanzienlijke verbetering in de functie en structuur van de nier. Voor het ingrijpen in de activiteit van beide groeifactoren zijn verschillende niveaus geïdentificeerd in hun pathofysiologische interacties met andere mediatoren, extracellulaire matrix en membraaneiwitten. Hoewel potentiële therapeutica zich nog in een vroege testfase bevinden, zijn de eerste klinische trials reeds geïnitieerd.

Perspectieven

De studies in dit proefschrift tonen aan dat CTGF een pathogenetische factor is in diabetische nefropathie en dat het meten van CTGF spiegels zou kunnen bijdragen aan vroegere identificatie van patiënten die een verhoogd risico hebben op een snelle achteruitgang van de nierfunctie. Momenteel lijkt klinische toepassing van zo'n test niet veel toegevoegde waarde te hebben, omdat plasma CTGF spiegels op dit moment niet direct beïnvloedbaar zijn en omdat, binnen de huidige therapeutische mogelijkheden, deze groep patiënten reeds optimaal behandeld wordt. Progressie naar eindstadium nierfalen is desondanks vaak onvermijdelijk. Wel zou het meten van plasma CTGF in de toekomst kunnen bijdragen aan het monitoren van de effectiviteit van behandelingsstrategieën die ook indirect de CTGF spiegels beïnvloeden. Bovendien is anti-CTGF therapie in ontwikkeling.
Onze preklinische studies tonen duidelijk aan dat CTGF een pathogenetische rol speelt in meerdere belangrijke aspecten van diabetische nefropathie. Er is eerder aangetoond dat zowel remming van TGF-β en VEGF signalering als toediening van BMP-7 leidt tot een vergelijkbare vermindering van structurele en functionele nierschade in experimentele modellen van diabetische nefropathie. De rol die CTGF heeft als modulator van deze en nog andere groeifactoren, zoals deels aangetoond in de studies beschreven in dit proefschrift, maakt anti-CTGF therapie een aantrekkelijke optie voor ontwikkeling van nieuwe behandelingsstrategieën.
Er zijn meerdere antilichamen ontwikkeld die gericht zijn tegen elk van de vier functionele domeinen van het CTGF molecuul. Het is nog niet bekend of deze antilichamen ook verschillende profielen hebben wat betreft in vitro en in vivo modulatie van de bioactiviteit van CTGF. Eerdere studies hebben aangetoond dat een anti-CTGF antilichaam dat speficiek bindt aan domein 2 van CTGF in staat is om schade in de nier en het cardiovasculaire systeem te remmen in experimentele diabetes. Hoewel domein 2 van CTGF geacht wordt betrokken te zijn bij modulatie van TGF-β en BMP signalering, is het mechanisme achter het therapeutische effect van dit antilichaam niet geheel opgehelderd. Het zal interessant zijn om de farmacologische profielen van deze en andere optionele anti-CTGF therapieën te vergelijken in toekomstige preklinische studies. Dit vooral in de context van fase 1b klinische trials met het anti-CTGF domein 2 antilichaam (FG3019) die recentelijk gestart zijn in diabetische nefropathie en andere fibrotische aandoeningen.

Nawoord

In dit proefschrift heb ik laten zien dat verstoring van de balans tussen CTGF en BMPs een belangrijke determinant is van overmatige fibrose, met name in diabetische nefropathie. Vorming van een litteken kan essentieel zijn na beschadiging, maar optimale regeneratie is afhankelijk van een juist evenwicht tussen profibrotische en antifibrotische factoren. Voor mij vormt dit de *yin* en *yang* van de respons op nierschade.

Acknowledgments:
Just Another Chapter in this Thesis?

Abstract

Introduction: The completion of a thesis is the most important goal for every PhD student. Previous studies have demonstrated that the contribution of the supervisor is essential in this process. However, writing a thesis involves numerous other factors, which may not be known to the outside world, but deserve to be acknowledged. The aim of the present study was to reveal which individuals have contributed significantly to the successful outcome of the thesis "CTGF and BMPs in diabetic nephropathy".

Materials and methods: Data concerning supervisors, colleagues, collaborators, friends, and family members were collected since 2003. Follow-up time of the study was 5.5 years. Univariate and multivariate analyses were used to identify those individuals whose contributions correlated with outcome of this thesis.

Results: The co-promotor proved to be the strongest predictor for successful outcome of the thesis (covariate-adjusted HR 24.06 [95% CI 13.11-31.08]). Other independent predictors were supervisors, colleagues, and internal and external collaborators. Furthermore, the support of family and friends was strongly correlated with the happiness of the PhD student ($R=0.77$, $P<0.01$). Three independent individuals were suspected of attempts to sabotage the confidence of the PhD student. These were deleted from the study due to lack of significance.

Conclusions: The present study has revealed several important individuals that determined the successful outcome of this thesis. No further studies will be needed to validate the conclusion that *Acknowledgments* are more than just another chapter in this thesis.

Introduction

It is a truth universally acknowledged that allowing a medical doctor to do experiments must cause disaster in the lab. Only the support of numerous people can prevent this and generate success instead. The aim of the present study was to reveal which individuals have contributed significantly to the successful outcome of this PhD project.

Materials and Methods

Categorization of subjects
All subjects who were related to the PhD student and his work between 2003 and 2008 were entered into the study. Subjects were categorized according to their role as co-promotor, supervisor, colleague, collaborator, friend, or family member. Secondary categorizations were made based on the nature of working relationship and geographical setting.

Definition of outcomes
Successful outcome of the study was defined by either professional or personal benefit for the PhD student. Professional benefit included co-authorship, difficulty of the experiments, novelty of the data, acceptability of the paper, and impact factor of the journal. Personal benefit was defined by happiness.

Statistical analysis
All data were logarithmically transformed before analysis. Spearman correlations and forward stepwise regression analysis were used to identify those individuals whose contributions correlated with successful outcome. Independent predictors were identified by Cox proportional hazard regression models. $P<0.05$ was considered significant.

Results

The co-promotor
The co-promotor was identified as the strongest predictor for successful outcome of the studies in this thesis (covariate-adjusted HR 24.06 [95% CI 13.11–31.08]). His combination of enthusiasm, sincerity, and profound interest in every aspect of life has resulted in both professional and personal benefit for the PhD student. Moreover, in addition to his role as co-promotor, he was able to act as mentor, inspirator, and motivator.

Roel, mijn dankbaarheid en waardering zijn niet in woorden uit te drukken. Vanaf het moment dat ik als student bij je aanklopte, ben je voor mijn ontwikkeling van onschatbare waarde geweest. Ik prijs me zeer gelukkig dat ik dit promotietraject onder jouw begeleiding heb kunnen afleggen. Ik ben trots deel uit te maken van je onderzoeksgroep en hoop dat onze unieke samenwerking nog lang zal voortduren. Ik wil jou, Pietia, Isa en Florian een fantastische tijd toewensen in San Francisco.

Amongst professors

The contribution of at least one supervisor is needed for completion of a PhD project. Also the residency in Pathology requires support from a supervisor. Although a book has been published in 1975 describing the bad reputation of professors, this thesis has identified nothing but positive aspects. Amongst professors from the department of Pathology at the UMCU, multivariate analysis has revealed three supervisors that contributed significantly to both the PhD project as well as to the residency.

Prof.dr. P.J. van Diest en prof.dr. G.J.A. Offerhaus, het is voor mij een grote eer dat ik jullie als promotoren heb. Paul, jouw onvoorwaardelijke steun heeft het mogelijk gemaakt dat ik opleiding en onderzoek kon combineren. Johan, jouw energie en lach zijn aanstekelijk. Dank je voor het ontfermen over de niergroep in die gevallen dat het nodig was.
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The kidney group

Each individual member of the kidney group was identified as a key player for outcome of this thesis. This proved to be not only the case for the present members, but also for two former PhD students, a post-doc, and three students.

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The department of Pathology

Review of the chapters in this thesis revealed that nearly all the work was performed at the department of Pathology. This department was shown to be the ideal work environment for both research and diagnostics. All separate subdivisions of the department, including staff and residents, MIP, MRL, histology, cytology, Biobank, and administrative and ICT support, were correlated with the activities of the PhD student/resident.

De combinatie van onderzoek en opleiding was voor mij nooit mogelijk geweest zonder de geweldige steun vanuit de afdeling. Omdat het mij niet lukt om een ieder persoonlijk bij naam te noemen, wil ik daarom op deze plaats mijn dank uitspreken aan alle medewerkers en ex-medewerkers van de afdeling Pathologie. Tijdens mijn werkzaamheden als arts-assistent en onderzoeker ben ik vrijwel met ieder van jullie in aanraking gekomen en dat contact heb ik veelal als zeer prettig ervaren. Ik kijk ernaar uit ook de komende jaren nog veel met jullie samen te werken.

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Internal and external collaborators

No statistical analysis was required to demonstrate that the contribution of internal and external collaborators was essential to the studies in this thesis. These collaborations consisted of the departments of Vascular Medicine and Nephrology of the UMCU, Nephrology of the UMC St Radboud, Pathology of the AMC, Hubrecht Laboratory, Developmental Biology of UCLA, Pathology of Oxford University, Molecular Biology of the Catholic University of Chile, FibroGen, Inc., and Steno Diabetes Center.

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Family and friends

Finally, the contribution of friends and family members was analyzed in conjunction with conventional factors. Although not significantly associated with the professional benefit of the PhD student, it was demonstrated that the support of family and friends was strongly correlated with the happiness of the PhD student ($R=0.77$, $P<0.01$).

Hans, Annemarie, Esther, Sam, Anneke, Alfred en Nienke, ik wil jullie hartelijk bedanken voor de hechte vriendschap en alle welkome afleidingen tussen mijn experimenten door. Alferso, geweldig dat we als vrienden nu samen onderzoek gaan doen. Jennifer en Steven, jullie aanwezigheid op de kamer heeft destijds voor veel gezelligheid en afwisseling gezorgd. Dit heeft mij op meerdere momenten veel goed gedaan. Tom, leuk dat ik jouw vriendschap te danken heb aan onze gezamenlijke tijd in het CTGF onderzoek.
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Conclusion

This chapter has demonstrated that successful completion of a PhD thesis requires the support of numerous people. Therefore, I conclude that *Acknowledgments* are more than just another chapter in a thesis.

List of Publications

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