Fibrogenesis in progressive renal disease

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Chapter 1

General Introduction

General introduction

Glomerulosclerosis results from an excess accumulation of extracellular matrix (ECM) molecules leading to glomerular scarring. Accumulation of ECM leads to progression of renal disease, a process called fibrogenesis. This progression is characterized clinically by loss of renal function. Glomerulosclerosis can be caused by several factors, which have been summarized in Table 1.

The aim of the work described in this thesis was to investigate the underlying molecular mechanisms of the development of glomerulosclerosis. Quantification of gene expression provides insight into the molecules and mechanisms involved in fibrogenesis. Animals developing glomerulosclerosis are being investigated as models for glomerulosclerosis in patients.

The introductory section for this thesis is structured as follows. After discussing the anatomy and function of the normal kidney, the role of the ECM in normal kidneys and during progression to glomerulosclerosis will be elaborated on. The increase of ECM observed in progression to glomerulosclerosis results from an altered balance between ECM production and degradation. In addition to quantitative changes, qualitative changes in the ECM production may also play a role in its accumulation. These processes are regulated by growth factors and cytokines. After giving an overview of animal models for glomerulonephritis, an introduction on diabetic nephropathy (DN) is provided. DN is a major complication of both type 1 and type 2 diabetes and is the most common cause of end-stage renal disease (ESRD) (1); thus, this introduction includes several factors involved in the progression of DN and a review of the most commonly used animal models for DN. To bring the molecular interactions into focus, an overview of the current knowledge of the role that growth factors, such as transforming growth factor-beta (TGF-β), connective tissue growth factor (CTGF), and vascular endothelial growth

Table 1. Causes of glomerulosclerosis

- Immune-mediated glomerulonephritis
- Metabolic disease
- Infection
- Drug induced nephrotoxicity
- Hemodynamic abnormalities
- Genetic
- Aging
- Idiopathic

factor (VEGF), play in the progression of DN is given. This overview is followed by a summary of mRNA detection methods and discussion of their effectiveness. The introductory section concludes with a description of the goals of the studies described in this thesis.

Anatomy and function of the kidney

The kidneys are organs that are specialized in maintenance of water and electrolyte balance. The human kidneys are located in the retroperitoneum and weigh 130–150 g each. The organs are encased in a capsule, which is surrounded by retroperitoneal fat. The hilum of the kidney opens onto the renal vessels, lymphatics, and ureter. The anatomic unit of the kidney is the

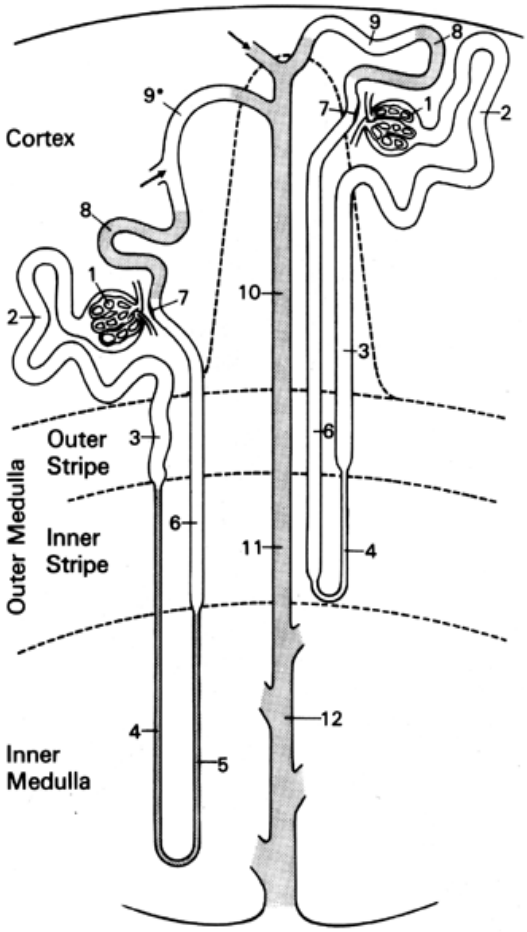

Figure 1. Schematic drawing of the nephron. Glomerulus (1). Proximal tubule (2-3). Descending thin limb (4). Ascending thin limb (5). Distal tubule (6). Macula densa (7). Distal tubule (8). Collecting duct (9-12).

nephron, which is composed of the glomerulus, proximal tubule, loop of Henle, distal tubule, and collecting duct (Fig. 1). Each kidney contains approximately 1.8 million nephrons (2).

Filtration of the blood plasma takes place in the glomerulus of the kidney. The glomerulus is composed of an afferent and efferent arteriole, the mesangium consisting of mesangial cells and ECM, and intervening capillaries lined by endothelial cells that cover the glomerular basement membrane (GBM). The outer surface of the capillaries, which is covered by glomerular epithelial cells (podocytes) (Fig. 2), is continuous with the epithelium of Bowman's space and the proximal tubule. Filtration of blood plasma takes place in the glomerular capillaries and is driven by the hydrostatic pressure of the blood flow. Filtration takes place at three different levels. First, it occurs at the level of the fenestrated endothelial cells, which are permeable to water and small solutes. The fenestrae themselves are too large to effectively filter macromolecules, but the endothelial cells are covered by a layer of negatively charged glycosylated macromolecules known as the glycocalyx (3). Haraldsson et al. (4) found that this negatively charged glycocalyx may play a role in glomerular size and charge selectivity.

The second layer is the GBM, which is a gel-like material consisting of 90% water (5). The structural integrity of the GBM is derived from a network of different ECM molecules, including

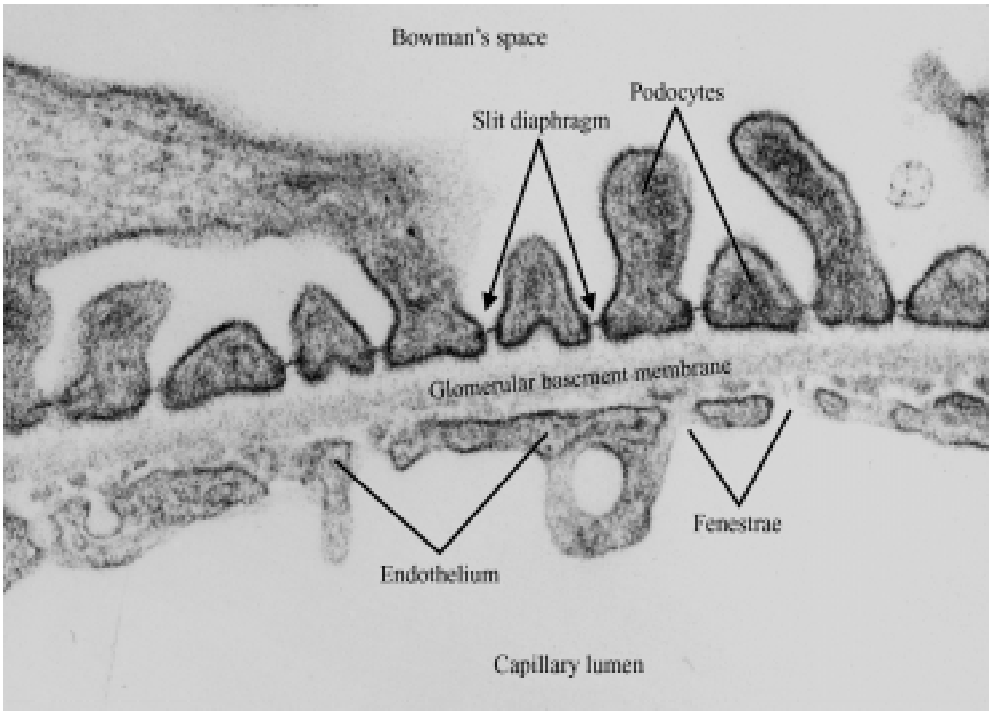

Figure 2. Schematic drawing of the filtration barrier.

type IV collagen, laminin, fibronectin, entactin, and heparan sulphate proteoglycans (6). The composition of the GBM and its molecular size and charge play an important role in permeability. The third barrier is the slit pore, located between the foot processes of the podocytes. The foot process is a contractile structure composed of many different molecules, including nephrin, CD2-associated protein, podocin, P-cadherin, densin, filtrin, actin, myosin, a-actinin, vinculin, and talin. These molecules are connected to each other or to the GBM at focal contacts such as the $\alpha_3\beta_1$-integrin complex (7). Podocytes also contribute to the specific size and charge characteristics of the glomerular filtration barrier, and damage to these cells leads to a retraction of their foot processes and proteinuria (8,9).

Extracellular matrix

In addition to their presence in the GBM, ECM molecules can be found in the mesangial area. Apart from its direct function in filtration, the GBM and the mesangial matrix serve as an anchoring place for glomerular cells through cell/matrix interacting sites such as integrins. The major components of the ECM in the glomerulus are collagens and laminins. Of all collagens, type IV is the primary one found here. It is encoded by six genetically distinct alpha-chains (alpha 1 through alpha 6) (10). Altered expression of collagen type IV alpha chains and laminin chains has been described in animal models for membranous nephropathy and lupus nephritis (11,12).

Progression of renal diseases as a result of disturbed ECM homeostasis

Most glomerulopathies are characterized by a decreased glomerular filtration rate (GFR) and proteinuria. Progression of renal diseases is morphologically characterized by the accumulation of ECM molecules in the glomerulus, the tubulointerstitial area, or both. Excessive accumulation of basement membrane, mesangial matrix, and interstitial matrix molecules are hallmarks of progression to glomerulosclerosis and interstitial fibrosis. A normal homeostasis of the ECM

Table 2. Causes of ECM accumulation

- Increased translation of ECM protein
- Increased transcription of ECM protein
- Reduced degradation of ECM protein
- Establishment of ECM binding sites on cells
- Establishment of novel binding (cellular/matrix) sites in ECM molecules via alternative splicing

is maintained by a continuous balance between the production and degradation of matrix molecules. Accumulation of ECM molecules in glomerulosclerotic and interstitial fibrotic lesions can be the result of increased local transcription or translation of ECM-encoding genes or RNA, trapping of ECM molecules from the circulation, or a diminished degradation of ECM proteins by matrix metalloproteases (MMPs) (Table 2). The MMPs belong to a large family of ECM-degrading enzymes, which include the interstitial collagenases (MMP-1, MMP-2, MMP-8, and MMP-13), stromelysins, gelatinases (MMP-2 and MMP-9), and elastases (13). Changes in MMP expression or activity may result into altered ECM turnover, which may lead to glomerulosclerosis. Cytokines such as TGF-β (14) and platelet-derived growth factor (PDGF) (15) can influence expression of ECM molecules and MMPs, thereby disturbing the balance between ECM synthesis and degradation. These cytokines can also lead to an altered composition of ECM molecules

Alternative splicing of ECM molecules in renal disease

Alternative splicing of ECM molecules can account for an alteration of the ECM composition by influencing the degradability of the matrix or by introducing matrix–matrix and matrix–cell interactions (Table 2). Splicing of mRNA takes place after DNA transcription. The encoding regions of most genes are split into segments (exons) separated by noncoding intervening sequences (introns). After transcription of DNA, the pre-mRNA molecules of most genes undergo further processing. This processing involves removal of the intron segments and rejoining of the remaining exon segments. The splicing of mRNA is mediated through a complex called the spliceosome, which consists of five types of small nuclear RNAs (snRNAs) and many other proteins (16) that assemble at splice sites. Each of the snRNA molecules is attached to specific proteins to form the spliceosome. The specificity of the splicing reaction is established by RNA-RNA base-pairing between the RNA transcript and snRNA molecules. In addition to physiologic RNA splicing, alternative splicing of RNA can occur. This process introduces splicing out or retention of exon sequences in addition to the intron sequences. With this mechanism, different cell types or environmental conditions can induce several types of mRNA molecules from a single gene.

Alternative splicing can be detected with reverse transcriptase polymerase chain reaction (RT-PCR) in combination with specific primers flanking the site where the splicing takes place. Separation on an agarose gel can discriminate between normally and alternatively spliced mRNA. Fibronectin is an example of alternative splicing of an ECM molecule. This glycoprotein plays a role in cell–matrix and matrix–matrix interactions and is found in the normal kidney. Its expression increases during glomerulosclerosis and interstitial fibrosis (17). Fibronectin is composed of a number of repeats of three different types and has several binding domains,

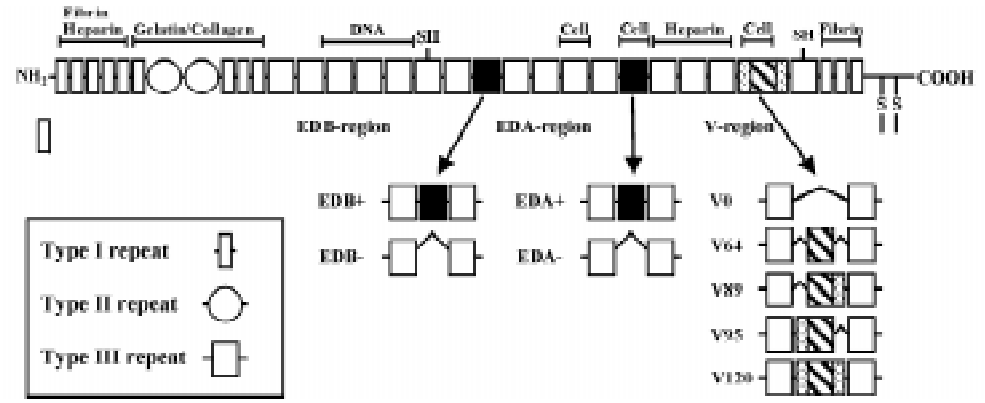

Figure 3. General structure of the fibronectin polypeptide. The fibronectin molecule consists of different repeats (types I, II, and III), several binding sites (fibrin, heparin, DNA, and cell), and three different sites that can be alternatively spliced (EDA, EDB, and V).

including collagen-, heparin-, and integrin-binding sites. Alternative splicing of certain domains within the fibronectin molecule can take place at three different regions, the EDA, EDB, or V regions. This alternative splicing can result in the retention of additional binding domains (Fig. 3) that play a role in biological processes, including maintenance of normal cell morphology, cell migration, cell differentiation, and cell remodeling (18). It has been shown that fibronectin proteins, including the EDA and V regions, are increased in the mesangium of nephritic rats. Coinciding with the up-regulation of the EDA and V120 isoforms, there was an increase in mesangial cell proliferation and in the number of infiltrating cells positive for a4ß1-integrin (a ligand for fibronectin) (19). Upon ischemic injury in rat kidneys, the expression of EDA-positive fibronectin increases dramatically in the renal interstitium and continues to be produced at high levels 6 weeks later. The V-region-containing fibronectin also increases in the interstitial space (20).

Animal models for glomerulosclerosis

Animal models of renal disease can be used as a tool to investigate the development and progression of human renal diseases. In this section, the animal models employed in the studies presented in this thesis will be discussed. First of all, anti-Thy-1 nephritis in rats, which is induced by a single injection of antibodies directed against the Thy-1 epitope on the glomerular mesangial cells, results in complement-dependent mesangial cell lysis, apoptosis, mesangial proliferation, and ECM deposition (21). Depending on the rat strain used, anti-Thy-1 glomerulonephritis either spontaneously resolves within several weeks or progresses to

glomerulosclerosis (22). Chronic serum sickness is a model for human membranous glomerulonephritis and can be induced by injection of human IgG in rats pre-immunized with human IgG (23). Immune complex glomerulonephritis is observed within a few weeks, accompanied by proliferation of mesangial cells and influx of macrophages. At the electron microscopic level, subepithelial and mesangial electron-dense deposits can be observed within the glomerulus (24). After 10 to 20 weeks, the rats develop mesangial matrix expansion followed by glomerulosclerosis and interstitial fibrosis.

In the mouse, chronic graft-versus-host (GVH) disease is used as a model for human membranous glomerulonephritis. Injection of parental-derived donor lymphocytes into F1-hybrids results in polyclonal B-cell activation (25). An array of autoantibodies is produced that can bind directly to the glomerular capillary wall. Immunofluorescence microscopy shows granular localization of immunoglobulins along the GBM in the mesangial area. The mice show global glomerulosclerosis 10 to 12 weeks after the induction of the disease (26).

Anti-glomerular basement membrane nephritis (anti-GBM) can be induced in mice through injection of rabbit anti-GBM antibodies. The antibody can be prepared by immunization of rabbits with mouse GBM (27). Animals develop glomerulonephritis and glomerulosclerosis within 14 days after injection.

Diabetic nephropathy

In several studies described in this thesis, we have focused our research on the development of glomerulosclerosis in patients with DN. DN is the most common cause of ESRD (1). After retinopathy, it is the most prevalent complication in patients with type 2 diabetes (28). Type 2 diabetes accounts for approximately 90% of all cases of diabetes. It is caused by a decreased response of liver and muscles to insulin or a disorganized insulin secretion by β-cells in the pancreas. As a result of these changes, patients experience high blood glucose levels. Diabetes mellitus can also lead to chronic vascular complications, which are the most important causes of morbidity and mortality. These consist of microvascular complications (microangiopathy) leading to retinopathy, neuropathy, and nephropathy, or macrovascular complications (macroangiopathy) leading to cardiovascular diseases. These vasculopathies are likely to result from endothelial cell dysfunction and damage caused by metabolic and hemodynamic factors (29).

DN is morphologically characterized by expansion of the mesangial matrix, thickening of the glomerular and tubular basement membranes, and glomerular hypertrophy (30). These features precede the development of glomerulosclerosis and interstitial fibrosis and the onset of the progression to ESRD. Nodular glomerulosclerosis, a characteristic pathological feature

first described by Kimmelstiel and Wilson (Kimmelstiel-Wilson lesion) (31), refers to the appearance of eosinophilic nodules at the periphery of the glomerulus. The nodules are the result of an expansion of the mesangium in combination with progressive occlusion of the glomerular capillaries (32). Red blood cell fragments in such lesions in combination with the presence of activated plasminogen activator inhibitor 1 indicate microvascular injury and mesangiolysis in DN (33).

Although many factors whose expression is associated with the progression of DN have been identified, the precise pathogenesis of this disease is still unknown. Several mechanisms by which diabetes can cause ESRD have been proposed (34-36). The most important postulated risk factors for DN are systemic hypertension, hyperglycemia, cigarette smoking, hyperlipidemia, duration of diabetes mellitus, dietary protein intake, and genetic predisposition (37,38). The following paragraphs provide an overview of several factors involved in the progression of DN.

Metabolic factors in the progression of DN

An increased blood glucose level is a major risk factor for the onset and progression of DN. Hyperglycemia mediates its effect in several ways. First of all, activation of the protein kinase C (PKC)-MAP kinase pathway plays a prime role in the development and progression of early tissue damage in DN (39,40). PKC has been implicated as a cause of altered renal blood flow (41) and of induction of several growth factors and ECM production in the diabetic kidney (42-47).

Progression of DN may be accelerated by the formation of metabolic derivatives such as oxidants and glycation products. Formation of reactive oxygen species due to metabolic changes in diabetes can contribute to the development of DN via oxidative stress and increased oxygen consumption (48).

Accelerated non-enzymatic glycation in diabetes, resulting from high glucose levels, is also linked to the pathogenesis of DN (49-52). This glycation, called the Amadori reaction, is a reaction between sugar molecules and polypeptides that irreversibly generates advanced glycation end products (AGEs) (53). AGEs can stimulate ECM production through activation of growth factors (54-58). There is also evidence that AGEs induce transition of tubular epithelial cells to myofibroblasts, which are major producers of ECM (59). Because of the slow turnover of ECM molecules, these proteins are highly susceptible to modification by AGEs, which possibly leads to decreased susceptibility of ECM proteins to degradation by MMPs (60). AGEs exert their effects on cells by interacting with specific cellular receptors, e.g., the receptor for advanced glycation endproducts (RAGE). This AGE-RAGE interaction can lead to cellular oxidative stress, resulting in several cellular responses, including activation of transcription factors.

Hemodynamic factors in the progression of diabetic nephropathy

Activation of the renin-angiotensin system (RAS) is one of the major mechanisms for changes in renal hemodynamics contributing to the progression of DN. Apart from the circulating RAS that regulates blood pressure, fluid-, and electrolyte balance, the kidney has an independently regulated local RAS (61). In the tubular cells and glomeruli, renin and angiotensinogen are expressed (62,63). High glucose activates the intrarenal RAS in cultured mesangial cells, resulting in decreased matrix degradation and increased matrix accumulation via induction of TGF-ß1 secretion (64). Streptozotocin (STZ)-induced diabetes in rats is accompanied by an increase of renin mRNA in the proximal tubules and by downregulation of cortical angiotensin receptors in the renal cortex (65).

The endothelin system is another mechanism in the kidney that regulates renal hemodynamics (66). Endothelin-1 (ET-1) is one of the most potent vasoconstrictors and acts as a paracrine and autocrine factor (67). ET-1 is present in the kidney, where it is secreted by mesangial (68), endothelial (69), and tubular epithelial cells (70). ET-1 production increases under high glucose conditions via TGF-ß stimulation (33,71,72) and by shear-stress as a result of glomerular hyperfiltration (73). ET-1 can stimulate cell proliferation and increase the expression of PDGF (74). Studies on the expression of ET-1 in diabetic animal models show conflicting results. Some studies show increased urine ET-1 levels in diabetic animals (75), while others found that renal ET-1 mRNA expression and protein were significantly reduced in diabetic kidneys (76). In hypertensive patients with type 2 diabetes, plasma ET-1 concentrations were increased compared to control subjects. This increase could be reduced by enalapril or nicardipine treatment (77).

Genetic predisposition to DN

Although blood glucose levels are often poorly controlled in diabetes mellitus, 60–70% of patients with type 2 diabetes mellitus never develop DN. Several studies show a higher incidence of DN in some families or ethnic populations (78-80). The prevalence of DN in diabetic patients with siblings with DN is about 50% higher than that in diabetic patients whose have siblings do not have DN (81). This observation suggests that a genetic predisposition underlies the progression of DN. Indeed, several single nucleotide polymorphisms (SNPs) associated with DN have been described. Two DN-related polymorphisms in the endothelial nitric oxide synthase gene have been identified (82). Others have found DN-related SNPs in the PKC-β1 gene (83), in the solute carrier family 12 member 3 gene (84), and in glutamine/fructose-6-phosphate amidotransferase-2 (85). Vardarli et al. found a strong linkage of chromosome 18q (LOD score of 6.1) with the occurrence of DN in Turkish families with type 2 diabetes mellitus (86). This

locus was confirmed by a genome-wide, gene-based SNP search for DN-related susceptibility genes in African-Americans (87).

Animal models for diabetic nephropathy

Several animal models for DN have been described in the literature. Mice and rats can be made diabetic by a single injection of STZ, providing a model for human insulin-dependent diabetes mellitus. There are several genetic knockout and transgenic mouse models for diabetes. These include the hypoinsulinemic non-obese diabetic (NOD) mouse, the Kkay mouse, the New Zealand obese mouse, the hyperinsulinemic ob/ob mouse, and the different strains of obese hyperinsulinemic db/db mice [summarized by Allen et al. (88)]. Each of model displays some morphological changes in the kidney that resemble those seen in diabetic patients. The db/db mouse model has been the most extensively investigated model for human DN. Db/db mice display substantial glomerular pathology, including mesangial matrix expansion and modest albuminuria. Diabetes can also be induced in PVG.RT1 rats. These relatively T-cell deficient rats develop diabetes after adult thymectomy and sublethal irradiation (89). More recently, OVE26 mice have been described as a transgenic model of severe, early-onset type 1 diabetes (90). These mice develop diabetes within the first weeks of life and survive well over one year without insulin treatment. The OVE26 mice show most of the characteristics of human DN, including glomerular hypertrophy and mesangial matrix expansion, followed by diffuse and nodular sclerosis, and tubulointerstitial fibrosis. The GFR of these mice increases significantly between 2 and 3 months of age and then decreases between 5 and 9 months.

Growth factors in renal diseases

TGF-β

Growth factors play a role in the progression of renal diseases. They mediate ECM homeostasis by increasing ECM production and diminishing degradation of ECM proteins. Growth factors can indirectly influence progression of renal disease via their proliferative and chemoattractive effects on cells that are involved in ECM homeostasis. TGF-β is one of the first and most extensively investigated growth factors in the progression of renal diseases. It is a 25-kD protein that is secreted in a latent form and requires cleaving before it can become active. The release of the latency-associated protein from the mature TGF-β protein by enzymes such as trombospondin and plasmin (91,92) is necessary for activation (93). TGF-β can directly induce ECM production in mesangial cells (94-97). TGF-β can also promote matrix accumulation via downregulation of MMPs or upregulation of tissue inhibitors of metalloproteinases (TIMPs) (98-100). TGF-β can also contribute to matrix expansion via induction of expression of receptors

for (circulating) matrix molecules. Kagami et al. have shown that TGF-ß can induce mRNA expression of α1β1 integrin in mesangial cells, resulting in a significant increase in adhesion of fibronectin, collagen I, and laminin to these cells (101).

In experimental animal models, Border et al. identified an important role for TGF-β in anti-Thy-1 nephritis (102). Administration of the natural TGF-β inhibitor decorin or antibodies against TGF-β to glomerulonephritic rats suppressed glomerular matrix production and prevented matrix accumulation (103,104). These findings have stimulated investigation of TGF-β in experimental renal diseases and human renal diseases. For example, TGF-β has been widely investigated in animal models for DN (58,105) and in anti-GBM nephritis (106,107). On the other hand, in mice suffering from chronic GVH disease, there was no evidence for a role of TGF-β in the development of glomerulosclerosis (108), indicating that TGF-β is not always necessary for the development of glomerulosclerosis.

Although there is considerable evidence for the role of TGF-β in the development of glomerulosclerosis in animal models, the evidence for its role in the development of glomerulosclerosis in patients is less convincing. Iwano et al., using quantitative RT-PCR, found higher expression of TGF-β1 mRNA in glomeruli of patients with DN (109). An increase of TGF-β expression has also been described in other renal diseases (110,111), including IgA nephropathy (112,113) and membranous nephropathy (114). On the other hand, in a study of patients with lupus nephritis and glomerulosclerosis, there was no increase of TGF-β mRNA in glomeruli (108). In biopsies from transplanted kidneys with acute rejection, higher levels of TGF-β were found compared to control tissue (115-118). Eikmans et al. showed that relatively high levels of TGF-β during acute rejection are associated with good prognosis (119). They hypothesized that TGF-β has beneficial effects during acute rejection through its anti-inflammatory actions or as an inducer of tissue repair. Until now, the precise role for TGF-β in progression of human renal disease has not yet been entirely clarified.

VEGF

VEGF, a highly conserved homodimeric glycoprotein with a relative molecular mass of 45 kD, is the only mitogen that specifically acts on endothelial cells. In addition to its potent mitogenic actions, VEGF also plays a prominent role in developmental angiogenesis (120). It can bind to Flk1, the major cell surface receptor for VEGF, which is exclusively expressed in endothelial cells (121). Hypoxia and hypoglycemia are major stimulators of VEGF expression (122). Hypoxia-induced transcription of VEGF mRNA is mediated, at least in part, by the binding of hypoxia-inducible factor 1 alpha (HIF-1α) to the VEGF promoter (123). More recently, hypoxia-induced c-Src thyrosine kinase activation was found to be another mechanism involved in VEGF induction (124). Other factors, including AGEs, PDGF, angiotensin II, nitric oxide,

prostaglandins, estrogen, and thyroid-stimulating hormone can also up regulate VEGF expression *in vitro* (125-130).

In the normal kidney, VEGF is present in podocytes and tubular epithelial cells, while it is absent in glomerular endothelial and mesangial cells (131). The specific mechanism by which VEGF may influence glomerular filtration is unknown. It has been suggested that VEGF is important for the maintenance of glomerular endothelial cells and that lowering local or circulating VEGF levels results in abnormal remodeling of the glomerular capillaries (132,133). Treatment of STZ-induced diabetic rats with monoclonal anti-VEGF antibodies decreases hyperfiltration, albuminuria, and glomerular hypertrophy (134). In the same model, VEGF was reduced in the glomeruli one week after induction (135). This reduction can be restored by treatment of the rats with insulin. Decreased VEGF expression was recently documented in the remnant kidney model, and treatment of these animals with VEGF reduces renal fibrosis (136). Administration of VEGF to rats after induction of anti-Thy-1 nephritis leads to enhanced endothelial cell proliferation and glomerular capillary repair (137). Proliferating endothelial cells were found in the mesangiolytic lesions and aneurysms. Thereafter, a new glomerular capillary network developed. In rats with anti-Thy-1 nephritis, a positive association was seen between impairment of vascular regeneration and the development of glomerulosclerosis (137-139). Studies in mice showed that both glomerular-selective depletion or overexpression of VEGF-A leads to glomerular abnormalities (140).

In glomeruli of patients suffering from DN, a decrease of VEGF mRNA has been described (132,133,141). At the same time, no correlation was found between renal function and circulating VEGF levels (142), indicating that local VEGF production seems to be more important for endothelial cell maintenance than circulating VEGF. In idiopathic membranous glomerulonephritis and in minimal change disease, expression of VEGF mRNA was considerably reduced compared to controls (143,144). In cell culture experiments on VEGF, opposing results have been obtained. Deposition of glycosylated IgA proteins result in reduced VEGF synthesis by mesangial cells (145), while high glucose can directly increase VEGF expression in mesangial cells via the PKC pathway (45,146). In the retinal pigment epithelial cell line, it has been shown that glycosylated albumin stimulates VEGF expression through an ERK-dependent pathway (147). In podocytes, high glucose induces activator protein-1–dependent transcriptional activity and expression of VEGF. AGE-induced activation of monocytes/macrophages resulted in augmented induction of VEGF and other angiogenic and inflammatory factors in these cells (148).

CTGF

CTGF is encoded by a 2.4-kb mRNA molecule. Northern blot analysis has shown that CTGF is

expressed in a wide variety of human tissues (149). It is a major chemotactic and mitogenic factor for connective tissue cells (150) and is associated with both systemic and localized fibrotic diseases and ECM synthesis (151-154). CTGF gene expression is increased in human fibroblasts upon stimulation with TGF-β but not with PDGF, epidermal growth factor, or basic fibroblast growth factor (bFGF) (155). Induction of CTGF expression in epithelial cells can occur directly via HIF-1α, independently of TGF-β (156). In addition to the fibrotic properties of CTGF, it is a potent angiogenic factor (157,158). It can regulate progression in invasive tumor angiogenesis by inducing expression of MMPs and by decreasing expression of TIMPs by vascular endothelial cells (159). The precise molecular mechanisms of CTGF's action as a growth factor are not completely known. Although much research has focused on unraveling its signaling pathway, a specific receptor has not yet been found.

In normal kidneys, CTGF mRNA is expressed mainly by visceral epithelial cells and to a lesser extent in parietal epithelial cells and interstitial fibroblasts (160). The role of CTGF in the normal kidney is still unclear, but we hypothesize that glomerular CTGF, in combination with other angiogenic factors such as VEGF, contributes to the normal maintenance of glomerular endothelial cells.

CTGF expression is increased in glomeruli and tubulointerstitial lesions from patients with glomerulonephritis, including IgA nephropathy, crescentic glomerulonephritis, lupus nephritis, and membranoproliferative glomerulonephritis. CTGF is involved in cellular proliferation and matrix accumulation (160-162).

Suppression subtractive hybridization techniques showed that CTGF is highly expressed in mesangial cells under high glucose conditions (163,164). This finding in mesangial cells was confirmed by others (165,166). Angiotensin II can induce CTGF in proximal tubular cells *in vitro* (71).

An increase in CTGF mRNA has been found in glomeruli of microalbuminuric and overt albuminuric patients compared to healthy and normo-albuminuric patients (167). In this study, it was proposed that CTGF mRNA, in combination with other glomerular mRNA markers chosen because of their pathogenetic relevance, may complement albuminuria and histology in predicting progression of DN. In diabetic NOD mice, a correlation was found between CTGF mRNA levels and the duration of diabetes. The most prominent mesangial CTGF immunostaining was seen in older animals (164). Other animal models for DN also showed an increase in CTGF mRNA and protein expression (165,168). Treatment of these animals with aminoguanidine or aspirin attenuated mesangial expansion suppressed CTGF induction and inhibited upregulation of TGF-β1 and fibronectin expression (169,170).

mRNA detection methods

To study the development of glomerulosclerosis, measurements of mRNA levels can be used as tools to investigate the molecular mechanisms behind this process. The genomic DNA itself does not direct protein synthesis, but uses mRNA as an intermediary molecule. The nucleotide sequence of the appropriate portion of the DNA molecule in a chromosome is first transcribed into mRNA. These mRNA molecules are used as templates for protein synthesis, a process called translation (Fig. 4). Proteins can be further modified posttranslationally. Quantification of gene expression levels in cells is important for investigating the gene patterns responsible for cell behavior during disease progression and response or resistance to treatment. Gene expression levels can also be used as a diagnostic tool (171) or as a predictor for disease outcome (119). Different techniques are available to measure gene expression levels.

Northern blot

One of the most conventional techniques for assessment of mRNA expression is Northern blot analysis. Negatively charged RNA is loaded into an agarose gel, and a negative current is applied to repel to molecules toward the positively charged electrical current at the opposite end of the gel. Because smaller RNA molecules move faster through the gel, RNA molecules are separated by size. The separated RNA molecules are transferred to a nylon filter, which is then

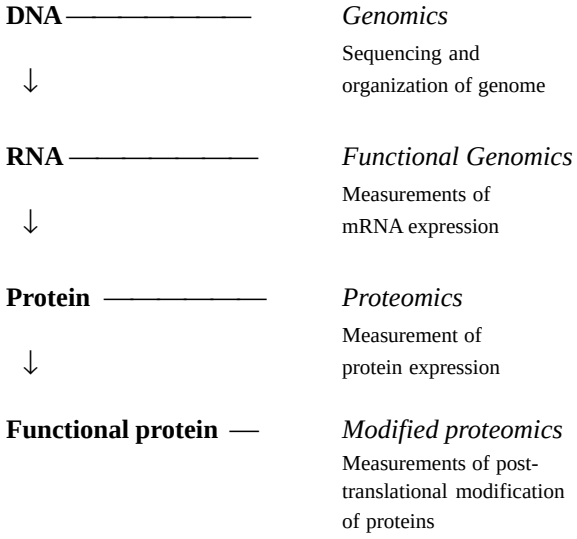

Figure 4. Transcription and translation: Genetic information is transcribed from DNA into mRNA and then translated from mRNA to protein. Proteins can be further modified posttranslationally to alter their function.

hybridized with a labeled, single-stranded DNA fragment encoding the gene of interest. The visualized DNA probe on the membrane is a measure for the expression level of the gene. With this labor-intensive technique, one can only quantify one gene at a time (Fig. 5). The sensitivity of Northern blotting is relatively low.

RNase protection assay

Another technique to quantify mRNA levels is the ribonuclease (RNase) protection assay (RPA). The principle of this method is based on protection against nucleases though specific binding of labeled antisense RNA probes. Subsequent treatment with RNase results in progressive cleavage of the overhanging mRNA sequence until the antisense RNA is hybridized only with the mRNA sequence of interest. Size-fractionation by denaturing gel electrophoresis can identify the sizes of different RNA probes. The amount of protected, labeled antisense probe corresponds with the relative gene transcription level of the mRNA sample. With this method, 5 to 20 genes can be quantified in one run.

RNA in situ *hybridization*

Hybridization of a gene-specific probe to tissue on the slide in combination with immunohistochemistry with cell-specific markers can give a detailed expression pattern showing which cells are expressing the particular mRNA transcript in the tissue. With the RNA *in situ* hybridization procedure, a labeled probe is hybridized to mRNA molecules present in frozen or paraffin sections. Optimal results are obtained with the use of an antisense riboprobe, which is generated by *in vitro* transcription of a cDNA cloned in a suitable vector. The labeled probe binds only to places in the tissue where the complementary mRNA is present.

Real-time PCR

PCR is based on the logarithmic amplification of specific DNA sequences. The first step in the analysis of mRNA is generation of cDNA copies from the mRNA molecules using reverse transcriptase in combination with Oligo-dT or random hexamer primers (172). The cDNA molecules can be used as templates for further amplification. One of the major problems with conventional PCR technique is the lack of correlation between the amount of cDNA input and the eventual amount of PCR product. This problem can be overcome by the use of quantitative real-time PCR (173). The principle of this method is continuous monitoring of the amplification with the use of a fluorescent probe that gives a signal when it binds to generated DNA. The real-time PCR machine can detect the fluorescent dye. This method makes quantitative comparisons of amplifications during the linear range possible. This method shows a high correlation with generated fluorescence and the amount of input cDNA and makes it possible

to quantify mRNA levels in samples containing 1 to 10 pg RNA.

Microarray analysis

Traditional efforts to understand disease pathogenesis have relied on a gene-by-gene analysis strategy, thereby limiting our abilities to devise novel therapeutic approaches. With the use of DNA microarray techniques (also known as DNA-chip technology), it is possible to generate mRNA expression profiles of thousands of genes within one sample. Two basic types of DNA microarrays are currently available: oligonucleotide arrays (174) and cDNA arrays (175). In the case of oligonucleotide arrays, 25-nucleotide–long fragments of known DNA sequences are synthesized *in situ* on the surface of the chip by using a series of light-directed coupling reactions similar to photolithography. By using this method, as many as 400,000 distinct sequences representing over 18,000 genes can be synthesized on a single 1.3-×-1.3-cm microarray chip. In the case of cDNA microarrays, cDNA fragments are placed onto the surface of a glass slide using a robotic spotting device. Both approaches involve hybridization of fluorescent labeled cRNA or cDNA material isolated from the tissue of interest (e.g., from renal biopsies or isolated glomeruli) to the microarray. The surface of the microarray slides is then scanned with a laser scanning device, which measures the fluorescence intensity at each position on the microarray. The fluorescence intensity of each spot on the array is proportional to the level of expression of the gene represented by that spot. This analysis results in an enormous amount of information, which needs careful indexing, storage, and organization. Computers are required for storage, distribution, and analysis of the data. The principal data banks holding such gene expression profiles are GenBank at the U.S. National Institutes of Health in Bethesda, Maryland, and the EMBL Sequence Data Base at the European Molecular Biology Laboratory in Heidelberg. These databases continuously exchange newly reported data and make them available via the Internet to biologists throughout the world. Computer programs are available to cluster genes in relation to each other or in relation to the disease. These techniques can be used to unravel the complexities of kidney diseases.

	Sensitivity	Input	No of genes	Quantitative	Tissue localization
Northern blot	+	10 µg	1	++	-
RPA	++	1-5 µg	5-20	++	-
Real time PCR	+++	1-10 pg	1	++	-
Microarray	+	1-20µg	$1\text{-}2*10^4$	+	-
RISH	+	1 slide	1	$\pm$	+++

Figure 5. Characteristics of different mRNA detection methods

Aims of this thesis

The central aim of the studies described in this thesis was to investigate the molecular mechanisms underlying the development of glomerulosclerosis in human renal diseases. To achieve this, measurements of mRNA steady-state levels can be used to obtain insights into the molecular processes occurring in the kidney. To study mRNA expression in the development of sclerosis in the glomerulus, it is important to isolate high-quality RNA from purified glomeruli. The aim of the first study was to assess the feasibility of isolating glomeruli from mouse kidneys and extracting their RNA (**Chapter 2**). Previous studies have shown that the ECM molecule fibronectin is abundantly present in glomerulosclerotic lesions. Fibronectin can be alternatively spliced at the EDA, EDB, and V regions, thereby generating different fibronectin isoforms. The aim of our second study was to investigate which fibronectin isoforms are present in biopsies from patients with kidney diseases leading to glomerulosclerosis (**Chapter 3**). To study the regulation of alternative splicing of fibronectin, we also investigated splicing at the mRNA level. For this, we used different animal models to test the presence of alternatively spliced EDA and EDB regions and correlated this with the presence of TFG-β. Cell culture experiments with rat mesangial cells were performed to study the effect of the cytokines TGF-β and IL-4 on the splicing of fibronectin mRNA *in vitro*. Finally, we measured mRNA levels of TGF-ß and fibronectin and the splicing pattern for fibronectin mRNA in human renal biopsies from patients developing glomerulosclerosis. These data were compared with data obtained from the animal models and cell culture experiments (**Chapter 4**).

In addition to assessing local fibronectin production, we also investigated trapping of plasma fibronectin from the circulation during the development of glomerulosclerosis. Earlier studies have shown that plasma fibronectin from the circulation can accumulate in glomerulosclerotic lesions. The aim of this study was to obtain more insight into the binding sites that play a role in the accumulation of fibronectin in pre-sclerotic glomeruli (**Chapter 5**). We also investigated the role of the heparin-binding domain on the binding of fibronectin in glomerulosclerotic lesions.

In **Chapter 6,** our goal was to identify genes and molecular pathways that are involved in the progression of DN. With the evolution of microarray techniques, it is possible to measure thousands of genes within one sample. We have used this powerful technique to measure gene expression levels in glomeruli from patients suffering from DN.

From the microarray studies, we found that several genes involved in angiogenesis were differentially expressed in patients with DN. We confirmed these data in biopsies from a larger patient group suffering from DN. The data were correlated with clinical and histological data of the patients to examine the role of angiogenic factors in different stages of the disease (**Chapter 7**).

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