Fibrogenesis in progressive renal disease

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Chapter 8

Summary and General Discussion

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Molecular biology offers new opportunities for experimental and clinical medicine. Promising clinical applications for patient care include identification of mRNA expression patterns (gene profiling) in diseased organs in correlation with diagnosis, prognosis, and responsiveness to different treatments. The development of novel technologies, such as microarray analysis and real-time PCR, allows study of gene expression networks, even in small renal biopsies. These technologies, in combination with the development of laser capture microdissection, enables specific gene expression analysis in a specific nephron segment.

Chapter 2 describes a method to isolate RNA from purified glomeruli. Because the size of mouse glomeruli is similar to that of mouse tubules, mouse glomeruli cannot be isolated using relatively simple sieving techniques. The development of a purification method for mouse glomeruli is mandatory for extraction of mouse glomerular RNA (1-3). One of the major problems with RNA isolation is that mRNAs are very sensitive to degradation by endonucleases and exonucleases. In normal living eukaryotic cells, several pathways have been identified that play a role in mRNA degradation. One of these involves the exosome, a multi-protein complex that degrades transcripts in the 3’ to 5’ direction and that contains nucleases related to the enzymes of the bacterial degradosome (4). Another mechanism is de-adenylation–dependent decapping. This decapping is triggered by removal of the poly(A) tail by exonucleases, followed by cleavage of the 5’ cap by decapping enzymes. After decapping, which normally prevents the mRNA from being translated, the mRNA undergoes rapid exonuclease digestion starting at the 5’ end. Normally, mRNA degradation is strictly regulated by this multi-protein complex. However, after cell death, endonucleases such as RNase E and RNase III, which make internal cuts in RNA molecules, and RNase II, which is an exonuclease that removes nucleotides in the 3’ to 5’ direction are released from their regulatory complexes and rapidly degrade mRNA. In this study, we tested the feasibility of using a novel glomerular isolation method in combination with RNA extraction. To optimize this procedure, the necessity of using RNase inhibitors was investigated in combination with different RNA isolation methods. We found that including RNase inhibitors was not necessary for obtaining intact mRNA, despite the fact that the whole procedure takes 2 to 3 hours. We conclude that the cells survive the isolation method and thus, the RNA is protected against degradation. Indeed, in another study we demonstrated that it is possible to culture viable mesangial cells from isolated mouse glomeruli (5). From the RNA extraction methods we have tested, the yield of the lithium chloride (LiCl)/phenol/chloroform method was about two to three times higher than the cesium chloride (CsCl) method (6). Based on the levels of intact 28S and the 18S ribosomal RNA bands after gel electrophoresis and signal intensity after hybridization with a collagen α1(IV) cDNA probe, the LiCl/phenol/chloroform

method gives the best RNA quality and quantity. For several years, kits based on phenol/ chloroform extraction without the use of LiCl have been available (e.g., Trizol®, Invitrogen). More recently, silica-gel spin columns were developed [e.g., RNeasy Mini columns (QIAGEN GmbH, Germany)], which can be used in combination with the stringency of guanidine-isothiocyanate lysis. We successfully used these kits with glomerular samples.

Chapter 3 describes the distribution of alternatively spliced fibronectin isoforms in human renal disease with developing glomerulosclerosis. From animal studies it is known that fibronectin is one of the major components of sclerotic lesions in the kidney (7). Furthermore, it was found that the extent of glomerular fibronectin deposition correlated with the severity of glomerular structural abnormalities in human renal diseases (8). The EDA exon in the fibronectin molecule was found more often in skin wound healing compared to normal skin (9). In Chapter 3 we describe our immunohistochemical investigations into the distribution of the various fibronectin isoforms in glomerulosclerotic lesions and in regions of tubulointerstitial fibrosis in several progressive human renal diseases. In areas of glomerulosclerosis and interstitial fibrosis, we found increased deposition of total fibronectin. EDA- and EDB-positive fibronectin isoforms were found in significantly increased amounts in glomerulosclerotic lesions compared to normal glomeruli. In areas with interstitial fibrosis, an increase in the amount of EDA-positive fibronectin was found, but no EDB-positive fibronectin was deposited in the fibrotic interstitium. These results show that in renal disease, oncofetal fibronectin (FDC6) and EDA- and EDB-containing fibronectin isoforms are upregulated at specific locations within the renal tissue, suggesting a specific pathogenic role for these fibronectin isoforms during disease development. There were no statistically significant differences in the expression of the various fibronectin isoforms among any of the patient groups. This finding suggests that excessive fibronectin accumulation is a final common phenomenon in the development of glomerulosclerosis and interstitial fibrosis.

Chapter 4 describes a study of alternatively spliced isoforms of fibronectin at the mRNA level in different animal models for immune-mediated glomerulosclerosis, and in human biopsies from patients developing glomerulosclerosis. Using cultured mesangial cells, we studied the effect of TGF-β and IL-4 on the splicing of fibronectin in the EDA and EDB regions. Using primers flanking the EDA or EDB regions, we performed RT-PCR on glomerular mRNA isolated from different animal models at several time points after induction of the disease, and on mRNA from renal biopsies from patients suffering from different glomerulopathies. Normal mice and rats did not express the oncofetal domains EDA and EDB. Induction of α-GBM nephritis, chronic serum sickness, or anti-Thy-1 nephritis resulted in inclusion of both the EDA and EDB domains in the fibronectin mRNA. However, induction of GvHD in mice had no effect on the splicing pattern of fibronectin mRNA. Culturing of glomerular mesangial cells in the presence of TGF-β led to inclusion of the EDA region, while IL-4–overexpressing mesangial cells showed

a significant decrease in EDA+ fibronectin mRNA levels. This decrease may have resulted from reduced TGF-β levels in the IL-4–overexpressing cells. A relation between the presence of TGF-β mRNA and EDA+ mRNA was also found in the animal models for glomerulonephritis and in patients developing glomerulosclerosis. This finding suggests that TGF-β plays a role in the induction of EDA+ fibronectin in the kidney and thus in the development of glomerulosclerosis. On the other hand, GVH-diseased animals develop glomerulosclerosis containing large amounts of fibronectin without demonstrable increase of TGF-β or EDA inclusion. In an earlier study we have shown that in these mice, the accumulation of fibronectin is a result of specific trapping of plasma fibronectin from the circulation (7). In addition, mice with constitutive transgenic expression of IL-4 show progressive glomerulosclerosis with mesangial accumulation of collagen types I, IV, and V in the absence of TGF-β1 upregulation. Although TGF-β and alternative splicing of fibronectin play a role in the progression to glomerulosclerosis, neither alternative fibronectin splicing nor high transcription levels of TFG-β appears to be a general prerequisite for the development of glomerulosclerosis.

In **Chapter 5**, trapping of plasma fibronectin from the circulation during development was investigated. Earlier studies have shown that plasma fibronectin can accumulate from the circulation in sclerotic lesions. To obtain more insight into the molecular binding sites that play a role in the accumulation of fibronectin in pre-sclerotic lesions, fibronectin was cut into different fragments. The different fragments were separated on a heparin affinity column, resulting in two batches of fragments with either low or high affinity for heparin. The fragments were labeled with fluorescein isothiocyanate (FITC) and injected into chronic GVH mice developing glomerulosclerosis. These fragments were also pre-incubated with heparin or N-desulfated non-anticoagulant heparin to investigate the role of the heparin binding site in the accumulation of fibronectin in sclerotic lesions. Whole, labelled plasma fibronectin (pFN) molecules were injected intravenously into mice 10 to 12 weeks after induction of GVH disease in mice and accumulated in the glomerulosclerotic lesions. The pattern of trapped fibronectin was comparable to that seen in PAS-positive glomerulosclerotic lesions. *Ex vivo* pre-incubation of pFN-FITC with heparin resulted in a reduced accumulation of pFN-FITC upon injection. Injection of pFN pre-incubated with non-anticoagulant, N-desulfated heparin also prevented the accumulation of pFN-FITC in the glomerular lesions. There was no difference between the accumulation of pFN-FITC pre-treated with heparin or non-anticoagulant heparin. Intravenous injection of the digested FITC-conjugated pFN fragments with low affinity for heparin resulted in accumulation in sclerotic glomeruli. This accumulation did not occur when the low-affinity fraction was injected in control mice. The FITC-conjugated pFN fragments with high affinity for heparin did not accumulate in glomeruli of GvHD mice. From these results, we conclude that the protective effect of heparin treatment may be the result of steric interference with the

specific binding sites, and not specifically because of blockade of the heparin binding site itself. Fibronectin can directly bind to ECM via its collagen binding sites. Secondly, fibronectin contains integrin binding sites for $\alpha5\beta1$ integrin that were increased in areas surrounding the glomerulosclerotic lesions. A third mechanism that may be involved in the accumulation of pFN in the glomerulus is activation of the coagulation system. However, based on the observation that both heparin or non-anticoagulant heparin can inhibit fibronectin accumulation, it is unlikely that this mechanism plays a role in the accumulation of fibronectin.

Chapter 6 shows the results of gene expression profiling of glomeruli from human kidneys with DN. DN is a major cause of morbidity in patients with type II diabetes (10). Although several factors, including high glucose, insulin, AGEs, and high blood pressure, may be involved in the progression of DN, the precise mechanism is still unclear. Therefore, we investigated the gene expression profile of glomerular RNA isolated from morphologically and functionally normal kidneys and from kidneys of patients with DN. About 100 genes were upregulated in the diabetic glomeruli, and about 500 genes were downregulated. One of the downregulated genes was VEGF, which is one of the most important factors in endothelial repair and angiogenesis. Considerable research has focused on the pathogenesis of endothelial dysfunction in patients with diabetes, but the exact role for VEGF during the development of DN has remained unclear until now.

In **Chapter 7**, we investigated expression of angiogenic factors identified by microarray analysis of kidneys from patients with DN or with normal kidneys. Endothelial cell loss and the role of VEGF in that loss determine development of renal disease and the progression to sclerosis. In human DN it has been suggested that VEGF is important in maintaining the glomerular endothelial cells and that a decrease in local VEGF levels accounts for abnormal remodeling of the glomerular capillaries (11,12). On microarray analysis, factors including CTGF, FGF2, and syndecan, which can induce new vessel formation (13,14), showed a decrease in patients with DN. We investigated the gene expression level of different angiogenic factors in renal biopsies from a larger patient group with DN. We found that VEGF and CTGF mRNA levels in both microdissected glomeruli and whole cortex were decreased in patients with DN compared to control kidneys. A negative correlation was found between glomerular VEGF mRNA and the extent of interstitial fibrosis. In other words, there is a relationship between progression of the disease and the reduction of glomerular VEGF mRNA. We also found a decreased number of endothelial cells both in glomerular and the tubulointerstitial tissue of patients with DN. From the results of this study, we speculate that loss of angiogenic factors contributes to the progression of DN. Podocyte injury resulting from the action of diabetic factors leads to loss of slit diaphragms and proteinuria. The resulting podocyte loss then leads to a reduction in expression of angiogenic factors, which are necessary for the normal

maintenance of the endothelial cells. The reduced endothelial cell maintenance in combination with endothelial cell dysfunction leads to a loss of endothelial cells followed by a loss of glomerular capillaries (Fig. 1).

On the other hand, it has been shown that urinary VEGF levels in patients with DN are increased. Although measurements of urine VEGF levels seem to be controversial in the literature, it has recently been demonstrated in a large patient group that urinary VEGF levels were increased and that this strongly correlated with 24-hour albumin excretion levels (15). In the same patients, plasma VEGF levels remained unchanged compared to a control group. The increase of urinary VEGF levels can be a result of VEGF synthesis in the kidney. The glomerular podocyte is the major site for renal VEGF synthesis, suggesting that the increased urinary VEGF excretion may be of glomerular origin. VEGF protein has also been found in proximal tubules of patients with late DN (16), suggesting that VEGF originating from renal tubules can also contribute to high levels of urinary VEGF. Based on our results and results from the literature, which show that both glomerular and tubularinterstitial VEGF mRNA levels decrease in DN (12,17), it is unlikely that the increased urinary VEGF levels result from increased VEGF production in the kidney. Because plasma VEGF levels remain unchanged in patients with DN, the explanations for higher urinary VEGF levels in patients with DN would be increased leakage of VEGF through the glomerular filtration barrier or a diminished absorption by proximal tubular

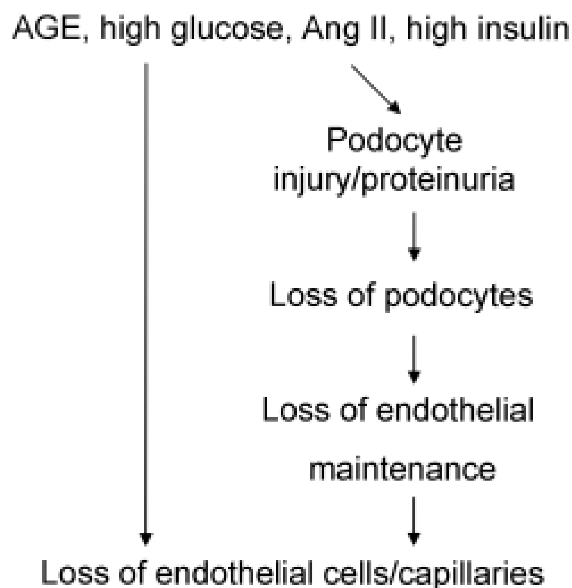

Figure 1. Partly speculative schematic illustration of endothelial cell/glomerular capillary loss in patients with diabetic nephropathy.

epithelial cells. This increased leakage of VEGF through the filtration barrier may also explain the correlation found between urinary VEGF levels and urinary albumin excretion in patients with DN. The activity of VEGF is highly regulated by sFlt-1, a naturally occurring soluble form of VEGF receptor. More studies are necessary to define the exact role of VEGF, together with sFlt-1 and other angiogenic factors, in the development and progression of DN

Further perspectives of gene expression profiling in renal diseases

With microarray technology, thousands of genes can be measured within one experiment, resulting in an expression profile of a biological sample. This information can be used for functional categorization of renal disease and may help in improving treatment of patients. Bioinformatics can also be used as a tool, leading to a better understanding of the molecular mechanisms that play a role in the progression of disease. With the interconnection of databases (e.g., NCBI, EMBL) containing information about gene sequences, chromosome locations, SNPs, gene ontology, and gene expression levels in different tissues and diseases, important information comes together and can be studied more easily. Also, programs such as Pathfinder® and the KEGG database are helpful tools to use in recognizing pathways involved in biological processes. A statistical global test for groups of differentially expressed genes within pathways is now available for the analysis of results obtained by microarrays (18).

In addition, the development of laser microdissection techniques (19) has enabled the separation of nephron segments in frozen or fixed sections. This development, in combination with new amplification protocols with a more than 1,000-fold linear amplification efficiency (20), gives us the opportunity for generation of nephron segment-specific gene profiles in frozen or paraffin-embedded patient material. Microfluidic biochips and nanotechnology-based biochips will gain importance as tools to study molecular mechanisms at the single-cell level (21).

The mRNA itself is not functionally active and must be translated into biologically active proteins. As with any multi-step biological process, eukaryotic translation can be regulated at various levels. The predominant step in the control of translation is ribosome binding. A large number of regulatory sequences have been identified in the 5' and especially in the 3' untranslated regions of mRNA. In addition, several factors, eukaryotic translation initiation factor 2 and 4E are the most important, play a crucial role in the regulation of translation initiation. Activation of these molecules is regulated by kinases (22), which are activated by environmental stress (23). These data imply that the number of transcripts for any mRNA molecule is not necessarily associated with the amount of the corresponding protein being translated. On the other hand, it is unlikely that cells expend a lot of energy on mRNA transcription without specific reasons. Although there are discrepancies between levels of

mRNA and the amount of protein, these differences can usually be explained by the occurrence of posttranslational processes, such as storage of proteins until they are necessary (for example coagulation factor VIII), or rapid protein degradation by proteolytic enzymes. Another explanation is slow protein turnover in normal maintenance of tissue, as occurs with ECM molecules. Although one can measure increased mRNA levels for these matrix molecules, it is possible to find no increase at the protein level because of a disturbed balance between production and degradation.

Within the framework of studying molecular biological processes, the logical step after genome-wide mRNA expression profiling is genome-wide protein expression profiling, also called proteomics. The goal of proteomics is a comprehensive, quantitative description of protein expression (24). The field of proteomics is growing rapidly. The rapid progress of proteomics over the past few years is a result of: (1) the completion of the human genome projects; (2) the improvement of gel-based protein separation techniques; and (3) the development of mass spectrometry (MS). One of the first applications of proteomics in renal disease was performed by Witzmann et al., who used the two-dimensional gel electrophoresis technique and MS analysis to study heat-shock and glucose-regulated proteins in the rat kidney (25). More recently, proteins derived from whole-kidney lysate of diabetic OVE26 transgenic mice were separated by two-dimensional PAGE and identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)-MS. Several proteins were differentially expressed in diabetic kidneys (26). Among these altered proteins, expression of monocyte/ neutrophil elastase inhibitor was increased, whereas elastase IIIB was decreased in diabetic kidneys.

When the expression of a gene is changed during disease, studies in animal models are necessary to confirm the functional role of that gene. Germ-line transgenic mouse models were developed to knock out or introduce a specific gene in the mouse genome (27). The disadvantage of this approach is that knockout of a gene often leads to (embryonic) lethality. To overcome these problems, an alternative approach is the use of conditional knockout strategies in combination with organ or cell specific transgene expression. Cell-type specific gene knockouts can be made with the use of Cre/lox system (28). This Cre recombinase system can be used in combination with an inducible cell specific promoter. Administration of a drug targeting the cre-construct in these mice induces excision of an integrated gene flanked by loxP sites in the selected tissues or cells (29).

Other methods for specific gene silencing are transfection of cells or tissues with RNA or DNA constructs that can turn off a gene. Several methods for transfecting cells are available. Commercial kits are available to transfect DNA constructs into cells *in vitro*. For *in vivo* applications, different methods have been described. Glomerular cells have been transfected

with the use of viruses such as hemagglutinating virus of Japan in combination with liposome transfection (30). Also, mesangial cells have been used to carry the gene construct into the renal glomerulus. The cDNA construct was introduced into cultured rat mesangial cells, and stably transfected vector cells were established (31). These cells were then delivered into the glomeruli of rats via the circulation. More recently, electroporation has been applied to introduce DNA into several organs. DNA/RNA constructs were injected into the left renal artery followed by electroporation of the injected kidney between a pair of tweezers-type electrodes (32). The advantage of electroporation compared to conventional methods is that this procedure is free from the oncogenicity, immunogenicity, and cytotoxicity of viral vectors or transfected cells. In addition, co-transfection with a mixture of different constructs may be achieved easily by electroporation (32).

Several methods can be used to turn off a specific gene. One of the oldest methods is the use of antisense RNA molecules to modulate the expression of selected genes. The principle is based on specific annealing of mRNA with complementary antisense RNA molecules resulting in double-stranded RNA that is no longer available for translation (33). The disadvantage of this technique is the short half-life time and the low efficiency of these antisense molecules (34). To overcome this problem, the use of double-stranded (ds) interfering RNA was developed. Compared to single-stranded RNA, dsRNA is relatively stable and does not require chemical modifications to achieve a satisfactory half-life. Long, double-stranded RNA molecules are processed by the endonuclease dicer into 21- to 23-nt small interfering RNAs (siRNAs), which specifically suppress gene expression in mammalian cell lines, including human embryonic kidney cells (35). Formation of dsRNAs results in loss of the corresponding mRNA. Binding to promoter and intronic sequences, results in largely ineffective transcription, and dsRNA can induce genomic methylation of sequences homologous to the silencing trigger, resulting in loss of transcription (36). It was found that the siRNA technique is at least 10 times more potent than conventional antisense RNAs in silencing a gene (37). Next to inactivation of mRNAs, exon skipping with the use of stabilized antisense oligonucleotides (AONs) can also be used to knock-down a gene function. Modulation of splicing by AONs that restored normal splicing by skipping exons with several different mutations in the ß-globin gene has been described by Dominski et al. (38). In addition to AON-mediated modulation of splicing, loss of gene function by skipping those exons that are necessary for a normal function of the gene has been proposed as a useful method of gene silencing.

Despite the ability to assess expression of thousands of genes simultaneously, which can give us insight into molecular biological processes at the single-cell level, and in spite of the current knowledge of many different pathways involved in disease, the precise mechanisms of the progression to glomerulosclerosis have not yet been fully clarified. We have to realize that

of the ~30,000 known genes in the human genome, 30–40% can be alternatively spliced at at least two different sites (39), which may theoretically result in about 100,000 different gene products that can also influence each other in a dose-dependent manner. This probability suggests that there is an enormous number of combinations that can influence the physiological state of a cell. More research is necessary, especially in the field of bioinformatics, that can recognize disease-specific gene expression patterns and improve our understanding of at least a part of the mechanisms behind the progression to glomerulosclerosis and interstitial fibrosis.

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