Protease-activated receptors in diabetic nephropathy and renal fibrosis

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Chapter 1

General introduction

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**General introduction**

In the Netherlands, 1.700.000 people suffer from chronic kidney disease, a condition in which fibrotic tissue builds up in the kidneys\(^1\). Independent of the underlying cause, chronic kidney disease will slowly progress to end stage renal disease\(^2\). At that stage, renal replacement therapy by dialysis or transplantation are the only therapeutic options left, which both have a huge social and economic impact. Apart from strict blood pressure control, there is no specific treatment to resolve or prevent the progression of renal fibrosis. In order to identify potential novel targets for therapeutic interventions, we need to better understand the development of renal fibrosis. With this knowledge we might be able to intervene earlier during disease progression and halt the progression of chronic kidney disease.

**The kidneys**

The kidneys, situated in the back of the abdominal cavity, are the organs responsible for maintaining homeostasis in the body. Each day, about 180 litres of blood gets filtered by the kidneys, from which about 1-2 litres of urine is produced. The kidneys not only filter waste products out of the bloodstream, but also regulate the blood volume and the amount of salts in the blood by active reabsorption of water, salts, proteins and amino acids.

The functional units of the kidneys are called nephrons (about 1.000.000 per kidney) and consist of a glomerulus (actual filtering unit) and a tubule (separated into the proximal tubule, the loop of Henle, and the distal tubule). All tubules end in the collecting duct, from which the pro-urine is transported via the ureter to the bladder. Blood enters the glomerulus via the afferent artery. Inside the glomerulus, fenestrated endothelial cells covered with a glycocalyx, the glomerular basement membrane and the podocyte foot processes form a filter that allows water and certain molecules to pass through. This so called ultrafiltrate is collected in Bowman’s space and drains down the tubular system. In the proximal tubule water and larger molecules get actively reabsorbed. Due to osmotic difference between the blood and the ultrafiltrate, water and salts get reabsorbed in the loop of Henle and the distal tubule.
Renal injury, repair and fibrosis

Fibrosis is the excessive accumulation of extracellular matrix (ECM). The origin of fibrosis lies in the wound healing process. Whenever a tissue gets injured, the wound healing process is initiated in order to replace the damaged tissue. The process of wound healing, independent of the underlying cause (which could for instance be an incision, chemicals, pathogens, or high blood pressure) or location of the injury, always follows four steps: haemostasis, inflammation, proliferation and remodelling (figure 1). When wound healing gets distorted – for instance when the initiating stimulus continues due to chronic inflammation or continuing hyperglycaemia—fibrotic scar tissue is formed, instead of regeneration of the old tissue.

Figure 1: Schematic representation of the wound healing process. Upon injury, the wound healing process gets initiated with haemostasis (1); activation of platelets and formation of a mature blood clot, followed by inflammation (2); migration of macrophages into the injured area that secrete cytokines and growth factors, proliferation (3); fibroblasts proliferate and secrete extracellular matrix proteins, and finally remodelling (4); repopulation of the injured area by the original cells and resolution of the extracellular matrix by metalloproteases.
(1) Haemostasis: Upon damage, the first action is to stop the bleeding. Whenever a blood vessel gets disrupted, circulating FVII comes in contact with tissue factor, which is expressed by the cells underneath the endothelium and gets exposed upon damage. This interaction initiates the coagulation cascade, by activating FVII (figure 2). Activated FVII (FVIIa) converts FX to FXa and FXa in turn converts prothrombin (FII) into thrombin (FIIa). Thrombin initiates amplification of the cascade by activating FV, FVIII and FXI. FXIa activates FIX and FIXa, together with FVIIIa, catalyses the activation of FX. FXa and FVa transform great amounts of prothrombin into thrombin, which in turn cleaves fibrinogen into fibrin. Fibrin mounts the activated platelets -that in the meantime have covered the wounded area- to form a mature blood clot.

To prevent excessive coagulation which may lead to thrombotic events, thrombin generation also leads to the activation of a feedback inhibition loop. When thrombin binds to thrombomodulin, protein C is converted to activated protein C (APC). APC in turn inactivates FVa and FVIIa, thereby preventing further thrombin formation. Antithrombin and tissue factor pathway inhibitor (TFPI) also inhibit coagulation proteases to prevent excessive coagulation.

(2) Inflammation: Secondary to haemostasis, macrophages and neutrophils migrate towards and into the injured site to clear apoptotic cells, debris from necrosis, and any source of infection. In addition, macrophages secrete cytokines and growth factors such as TGF-β, CTGF, MCP-1, TNF-α and IL1β that stimulate further wound healing.

(3) Proliferation: fibroblasts are attracted to the injured site where they proliferate, get activated and produce extracellular matrix that provides structure to the wounded area. Moreover, resident epithelial cells may transform into (myo)fibroblasts via a process called epithelial-to-mesenchymal transition (EMT). Upon TGF-β stimulation, tubular epithelial cells lose their epithelial phenotype and transform into mesenchymal cells expressing myofibroblast proteins such as α-SMA and vimentin.
Figure 2: The coagulation system Coagulation cascade (black/bold), feedback amplification loop (blue), feedback inhibition (red), haemostatic inhibition (green) and fibrinolysis (yellow). Orange arrows indicate changes upon diabetes and underlined proteases are known to activate protease-activated receptors.

4) Remodelling: the original cells will repopulate the injured site via proliferation and differentiation and finally increased production of matrix metalloproteases leads to resolution of the ECM. A dysregulated balance between extracellular matrix production and resolution results in accumulation of extracellular matrix. In renal fibrosis, extracellular matrix accumulates inside the glomeruli (glomerulosclerosis) and/or around the tubules (interstitial fibrosis), damaging the original structure and causing loss of renal function.

There are several forms of chronic kidney disease, which –independent of the primary cause- progress via renal fibrosis to end stage renal disease. Examples of chronic kidney diseases include: Glomerulonephritis, polycystic
kidney disease, obstructive nephropathy, hypertension and diabetic nephropathy. Almost half of all patients with chronic kidney disease suffers from diabetes, making diabetes the biggest risk factor for developing end stage renal disease (figure 3).

**Figure 3: Primary causes of kidney failure.** Source: United States Renal Data System: 2013 USRDS annual data report

**Diabetic nephropathy**

Diabetic nephropathy develops in about 30% of diabetic patients suffering from type 1 or type 2 diabetes. According to an estimation of the world health organisation, 422 million people were diagnosed with diabetes in 2014. In the United states alone, the number of diabetes patients has increased from 5 million in 1980, to 21 million in 2010. Although this number is more or less stable over the last 5-10 years, type 2 diabetes is diagnosed in a constantly younger population, while the life span of diabetic patients is increasing due to better management of glucose levels and hypertension. These facts predict an increased average duration of diabetes and thus an increased population at risk for developing diabetic nephropathy. As about 90% of the diabetic patients suffers from type 2 diabetes, which is often accompanied with obesity, hyperinsulaemia and/or hypertension, it is of great importance to also take these factors into account in modelling diabetic nephropathy.

Diabetic nephropathy is a slowly progressing disease that arises 10-25 years after the onset of diabetes. Generally, renal failure gives complaints only at a very late stage, when hardly anything can be done to delay further progression. In order to follow the progression of diabetic nephropathy, renal
function of diabetic patients is regularly. Five stages have been defined based on the glomerular filtration rate (GFR) and urinary albumin excretion (UAE; table 1) in order to monitor disease progression.

Initially, due to increased intraglomerular pressure, the glomerular filtration rate will rise (hyperfiltration; stage 1). To cope with ongoing intraglomerular pressure, mesangial cells proliferate and increase the production of extracellular matrix proteins, resulting in glomerular basement membrane (GBM) thickening and mesangial expansion (stage 2). Podocytes get damaged and detach from the basement membrane, resulting in leakage of proteins such as albumin into the urine (microalbuminuria; stage 3). While the disease progresses, more and more albumin will get excreted via the urine (macroalbuminuria; stage 4). Meanwhile the glomerular filtration rate declines, deteriorating kidney function (stage 5). Once the glomerular filtration rate drops below 15 ml/min, renal replacement therapy is necessary to prevent waste build-up in the body and consequent death.

The rise of blood glucose levels characteristic for diabetes mellitus, leads to the formation of advanced glycosylated end products (AGEs). AGEs are proteins, lipids or nucleotides to which excess circulating glucose is bound. AGEs may cause damage to blood vessels and the glycocalyx, initiating the wound healing response.

Table 1: Disease progression in diabetic nephropathy (GFR: glomerular filtration rate, UAE: urinary albumin excretion, GBM: glomerular basement membrane)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Manifestation</th>
<th>GFR (ml/min)</th>
<th>UAE (mg/day)</th>
<th>diabetes duration (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hyperfiltration</td>
<td>&gt;90</td>
<td>&lt;30</td>
<td>0-5</td>
</tr>
<tr>
<td>2</td>
<td>GBM thickening and mesangial expansion</td>
<td>&gt;90</td>
<td>&lt;30</td>
<td>5-10</td>
</tr>
<tr>
<td>3</td>
<td>Microalbuminuria</td>
<td>60-89</td>
<td>30-300</td>
<td>10-15</td>
</tr>
<tr>
<td>4</td>
<td>Macroalbuminuria</td>
<td>15-59</td>
<td>&gt;300</td>
<td>15-25</td>
</tr>
<tr>
<td>5</td>
<td>ESRD</td>
<td>&lt;15</td>
<td>&gt;300</td>
<td>25-30</td>
</tr>
</tbody>
</table>
Diabetes and coagulation

Besides high levels of AGEs, diabetes is associated with increased coagulation. Several coagulation factors are upregulated, resulting in increased plasma thrombin-anti-thrombin complexes (TAT) and soluble tissue factor (sTF) levels. As depicted in figure 1 (orange arrows), coagulation factor II (thrombin), VII and VIII, tissue factor, PAI-1 and fibrinogen levels are upregulated in diabetic patients\(^9,10\). Hyperinsulemia, which accompanies hyperglycemia in type 2 diabetes leads to elevated PAI-1 levels\(^11\).

With this hypercoagulable state during diabetes, it is tempting to speculate that anti-coagulant treatment may have beneficial effects on preventing the development of diabetic nephropathy. Several anticoagulant treatment strategies have indeed been investigated in the setting of diabetic nephropathy\(^12\). Treatment with sulodexide (a low molecular weight heparin, mixed with dermatan sulphate) for 9 months resulted in decreased albuminuria in Otsuka Long Evans Tokushima Fatty (OLETF) rats compared to untreated controls\(^13\). Also in diabetic patients, treatment with sulodexide has been reported to improve albuminuria\(^14\). Losartan (an angiotensin receptor blocker with antiplatelet and anticoagulant properties) treatment in wistar rats resulted in reduced segmental glomerulosclerosis upon streptozotocin-induced diabetes\(^15,16\) and a novel coumarin-aspirin compound XLF-III-43 was also reported to diminish mesangial expansion and glomerulosclerosis in diabetic rats, although the XLF-III-43 treated rats had reduced glucose levels compared to untreated controls\(^17\).

Several other studies, reported no beneficial effect of anticoagulant treatment in diabetic nephropathy. In one study, diabetic wildtype mice were injected daily with low-molecular-weight heparin. As expected, low-molecular-weight heparin normalized markers of coagulation (i.e. Thrombin-anti-thrombin, D-dimer and renal fibrin deposition). However, it did not protect against diabetic nephropathy. Indeed, albuminuria, kidney weight, histological PAS scores and glomerular size were similar in saline and low-molecular-weight heparin treated diabetic mice\(^18\). In line, the direct thrombin inhibitor hirudin did also not affect diabetic nephropathy (i.e. albuminuria and extracellular matrix deposition)\(^19\). Also multiple clinical trials using Sulodexide as an intervention failed to find significant improvement of albuminuria\(^20,21\).
Taken together, although some studies had promising results, enthusiasm for anticoagulant treatment in the setting of diabetic nephropathy faded due to these conflicting results.

**Protease-activated receptors**

Apart from their role in haemostasis, coagulation factors are also involved in several (patho-) physiological processes, such as proliferation, migration and chemokine secretion. The notion that coagulation factors may influence cellular processes apart from their function in the coagulation system, sparked novel interest on the hypothesis that coagulation factors may play a critical role in the development of fibrosis and diabetic nephropathy.

In 1991 the thrombin receptor was identified as the first protease-activated receptor (PAR). Protease-activated receptors are seven-transmembrane receptors that signal via G-proteins. Unlike other G protein-coupled receptors, PARs are activated by proteolytic cleavage of the N-terminus, revealing a tethered ligand that auto-activates the receptor. There are four PARs known today (PAR 1-4), which are all activated via proteolytic cleavage, have distinct tethered ligand sequences and can function as protomers, as well as heterodimers. Multiple proteases can activate a PAR, leading to different conformational changes, downstream signalling and cellular effects. For instance in the kidney, APC-induced PAR-1 activation leads to a decrease of podocyte apoptosis, whereas thrombin-induced PAR-1 activation leads to an increase of podocyte apoptosis. Apart from revealing a tethered ligand, some proteases (for instance cathepsin G) completely cleave off the tethered ligand, resulting in disarming of the receptor. Moreover, short synthetic agonist peptides that resemble the tethered ligand sequences can activate the receptor without proteolytic cleavage. Table 2 gives an overview of the known proteases that cleave PAR-1 and PAR-2 (the two PARs that will be discussed in this thesis). Among the list are coagulation factors, matrix metalloproteases (MMPs), Kallikreins (KLKs), granzymes and neutrophil related proteases.

PAR-1 and PAR-2 are key players in fibroproliferative disease. Activation of PARs leads to activation of inflammatory cells such as monocytes, T lymphocytes and mast cells, and proliferation of other cell types,
including epithelial cells, fibroblasts, and mesenchymal cells\textsuperscript{26-28}. In experimental animal models, PAR-1 deficiency has been shown to limit liver\textsuperscript{29}, lung\textsuperscript{30} and skin\textsuperscript{31} fibrosis. Indeed, PAR-1 deficient mice are (partly) protected against carbon tetrachloride-induced liver fibrosis\textsuperscript{29} and bleomycin-induced lung fibrosis\textsuperscript{32}. PAR-2 deficient mice are also protected against bleomycin-induced lung fibrosis (over 70\% reduction in fibrotic area)\textsuperscript{33}. Moreover, pharmacological inhibition of PAR-1 using p1pal12 reduced bleomycin-induced pulmonary fibrosis\textsuperscript{34}, confirming the important role of PAR-1 in fibrotic disease.

In the kidney, PAR-1 is widely expressed by glomerular endothelial cells, mesangial cells, tubular cells and podocytes\textsuperscript{81,82}. PAR-2 is also widely expressed in the kidney, mainly by epithelial cells, mesenchymal cells, interstitial fibroblasts and infiltrating renal inflammatory cells\textsuperscript{83,84}. PAR-1 is upregulated in glomeruli of diabetic mice and in a murine model of crescentic glomerulonephritis, the use of PAR-1 knockout mice effectively reduced crescent formation, glomerular inflammatory cell infiltration, and serum creatinine concentrations. In patients with chronic allograft nephropathy, tubular expression of PAR-1 is markedly up-regulated and correlates with interstitial fibrin deposition, the degree of tubulointerstitial fibrosis, and urinary excretion of TGF-β\textsuperscript{20}. Moreover, PAR-2 expression is up-regulated in the kidney of patients with IgA nephropathy, in a mouse model of unilateral ureteral obstruction and in the kidney of rats subjected to experimental glomerulonephritis\textsuperscript{83,84}.

Overall, these data suggest that PARs may indeed be of major importance in orchestrating the fibrotic response in renal disease but clear proof is lacking. This thesis focusses on the role of PARs in diabetic nephropathy and renal fibrosis in order to better understand its pathogenesis ultimately resulting in the identification of novel potential targets to treat this devastating complication of diabetes.
<table>
<thead>
<tr>
<th>Protease</th>
<th>Action</th>
<th>Known function in kidney?</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAR-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombin(^{23,35})</td>
<td>Activation</td>
<td>Prevents (50 pM) or increases (20 nM) glucose-induced podocyte apoptosis in a concentration dependent manner. (^{19})</td>
</tr>
<tr>
<td>FXa (^{36,37})</td>
<td>Activation</td>
<td>unknown (^{36})</td>
</tr>
<tr>
<td>APC (^{18})</td>
<td>Activation</td>
<td>prevents glucose-induced podocyte (but not mesangial cell) apoptosis (^{18})</td>
</tr>
<tr>
<td>Plasmine (^{38,39})</td>
<td>Activation/inactivation</td>
<td>Induces EMT in cortical tubular epithelial cells (^{39})</td>
</tr>
<tr>
<td>MMP-1 (^{40,41})</td>
<td>Activation</td>
<td>unknown</td>
</tr>
<tr>
<td>MMP-9 (^{42})</td>
<td>Activation</td>
<td>unknown</td>
</tr>
<tr>
<td>MMP-13 (^{43})</td>
<td>Activation</td>
<td>unknown</td>
</tr>
<tr>
<td>Granzyme K (^{44})</td>
<td>Activation</td>
<td>unknown</td>
</tr>
<tr>
<td>PRSS3/ mesotrypsin/trypsin IV (^{45-47})</td>
<td>Activation</td>
<td>unknown (^{45})</td>
</tr>
<tr>
<td>Proteinase 3 (^{48,49})</td>
<td>Activation/inactivation</td>
<td>unknown</td>
</tr>
<tr>
<td>Neutrophil elastase (^{48,49})</td>
<td>Activation/inactivation</td>
<td>unknown</td>
</tr>
<tr>
<td>KLK1 (^{50-52})</td>
<td>Activation/inactivation</td>
<td>unknown</td>
</tr>
<tr>
<td>KLK4 (^{53})</td>
<td>Activation</td>
<td>unknown</td>
</tr>
<tr>
<td>KLK6 (^{54,55})</td>
<td>Activation</td>
<td>unknown</td>
</tr>
<tr>
<td>KLK14 (^{54,56,57})</td>
<td>Activation/inactivation</td>
<td>unknown</td>
</tr>
<tr>
<td>Granzyme A (^{58})</td>
<td>Activation</td>
<td>unknown</td>
</tr>
<tr>
<td>Granzyme B (^{59})</td>
<td>Activation</td>
<td>unknown</td>
</tr>
<tr>
<td>Cathepsin G (^{60,61})</td>
<td>Activation/inactivation</td>
<td>unknown</td>
</tr>
</tbody>
</table>
Table 2 (cont.) List of proteases that can activate or disarm (inactivate) PAR-1 and PAR-2.

<table>
<thead>
<tr>
<th>Protease</th>
<th>Action</th>
<th>Known function in kidney?</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAR-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TF/ FXa/ FVIIa 36,37,62,63</td>
<td>Activation</td>
<td>unknown</td>
</tr>
<tr>
<td>Trypsin</td>
<td>Activation</td>
<td>Induces ERK phosphorylation and fibronectin production in proximal tubular epithelial cells 64</td>
</tr>
<tr>
<td>Mast cell tryptase 65</td>
<td>Activation</td>
<td>unknown</td>
</tr>
<tr>
<td>Matriptase 66</td>
<td>Activation</td>
<td>unknown</td>
</tr>
<tr>
<td>HAT 67</td>
<td>Activation</td>
<td>unknown</td>
</tr>
<tr>
<td>PRSS3/ mesotrypsin/ trypsin IV 45,46,68</td>
<td>Activation</td>
<td>No, mesotrypsin does not activate PAR-2 on renal epithelial cells, but does activate PAR-2 on brain cells. 45,68</td>
</tr>
<tr>
<td>TMRPRSS2 69</td>
<td>Activation</td>
<td>unknown</td>
</tr>
<tr>
<td>KLK2</td>
<td>Activation</td>
<td>unknown</td>
</tr>
<tr>
<td>KLK4 54,70</td>
<td>Activation</td>
<td>unknown</td>
</tr>
<tr>
<td>KLK5 54,71</td>
<td>Activation</td>
<td>unknown</td>
</tr>
<tr>
<td>KLK6 54,55</td>
<td>Activation</td>
<td>unknown</td>
</tr>
<tr>
<td>KLK14 54,72</td>
<td>Activation</td>
<td>unknown</td>
</tr>
<tr>
<td>Plasmin 73</td>
<td>Inactivation</td>
<td>unknown</td>
</tr>
<tr>
<td>Protease 3 74,75</td>
<td>Inactivation</td>
<td>PR3 protects the endothelial barrier 74,76</td>
</tr>
<tr>
<td>Cathepsin G 77</td>
<td>Inactivation</td>
<td>unknown</td>
</tr>
<tr>
<td>elastase 77,78</td>
<td>Inactivation</td>
<td>unknown</td>
</tr>
<tr>
<td>Chymase 79</td>
<td>Activation</td>
<td>Chymase increases albumin permeability 79</td>
</tr>
<tr>
<td>acrosin 80</td>
<td>Activation</td>
<td>unknown</td>
</tr>
</tbody>
</table>
Outline of the thesis

To investigate whether PAR-1 and PAR-2 play a role in the development of diabetic nephropathy, we studied diabetic nephropathy development in wild type, PAR-1 and PAR-2 deficient mice. Chapter 2 describes our findings in PAR-1 deficient mice and chapter 3 covers our finding in PAR-2 deficient mice. We found that PAR-1 deficiency protected diabetic mice from developing diabetic nephropathy, whereas PAR-2 rather acted as a double edged sword; resulting in both beneficial and detrimental effects. Based on these results we continue in chapter 4 with treating diabetic mice with the PAR-1 inhibitor vorapaxar. In chapter 5 we describe the effect of vorapaxar treatment in a mouse model for type 2 diabetes. As PAR-1 has many ligands that trigger both beneficial and detrimental signalling, in chapter 6 we attempted to identify the protease(s) responsible for the detrimental effect of PAR-1 activation during diabetic nephropathy. Finally, to explore the role of PAR-1 in the development of tubulointerstitial fibrosis, we subjected PAR-1 deficient mice to unilateral ureter obstruction (chapter 7). The thesis ends with chapter 8, a general discussion providing the general conclusions of this thesis and its implications.
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