The therapeutic potential of adenoviral gene therapy and angiotensine-(1-7) in proteinuric kidney disease
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Chapter 7

Summary, discussion and future directions
Chronic kidney disease (CKD) comprises of a collection of diseases with various aetiologies, which all tend to progress towards end-stage renal disease and ultimately require renal transplantation or dialysis. End-stage renal disease is a serious condition with high morbidity and mortality. Consequently, it is of pivotal importance to halt the progression of CKD. However, the best available therapy, inhibition of the renin-angiotensin-aldosterone system (RAAS), can at best slow down the progression of CKD and RAAS inhibition is not effective in all patients. Therefore, there is an urgent need for additional therapeutic strategies. Gene therapy holds the promise to offer such new approaches. Not only may gene therapy resolve the issue of therapy resistance to the currently available drugs, which is still a major problem in the field of nephrology, it also may address issues such as systemic side effects of drugs that need to act only in the kidney. Furthermore, gene therapy may offer new opportunities to explore the therapeutic potential of novel peptides of which nonpeptide agonists and/or antagonists are not yet available.

To establish which vector is the most effective in transducing genes into the kidney, the currently available literature on renal gene therapy is reviewed in Chapter 2. Adenoviral gene transduction is the most extensively studied strategy and appears to be among the most effective vectors for the kidney. Administration through the renal artery seems to be the most efficient approach. Transgene expression was found to localise in blood vessels, glomerular cells or interstitial cells, depending on the injection technique. With clamping of the renal vein, transduction is mainly found in blood vessels and interstitial cells, whereas slow infusion or perfusion with adenovirus results in glomerular expression. However, prolonged exposure following clamping, perfusion or slow infusion is necessary to achieve a higher expression. Other routes of administration result in transduction of other renal structures, including pyelum and tubules. In the near future, the development of less immunogenic adenoviruses may enhance the duration of transgene expression.

Because adenovirus transduction can be substantially enhanced by insertion of an integrin-binding RGD-motif, we studied whether an RGD-modified adenovirus (Ad-RGD) is suitable as vector for efficient and selective transduction of healthy and proteinuric kidneys. In both healthy and adriamycin-nephrotic rats, Ad-RGD resulted in strong transgene expression in the infected kidney, as described in Chapter 3. Transgene expression was localised mainly in interstitial (myo)fibroblasts. Expression was still detectable at 14 days after infection. No differences in level, time course or localisation of infection were observed between control and nephrotic rats. The administration of Ad-RGD did not result in any renal damage, but substantial liver tropism of Ad-RGD was observed. Therefore, Ad-RGD proves to be an efficient vector for gene therapy after injection in the renal artery, and targets interstitial fibroblasts both in healthy and nephrotic kidneys. The fibroblast-specificity offers new perspectives for Ad-RGD for treatment of renal fibrosis. This is the first time that efficient and selective transduction of renal fibroblasts has been described not only in healthy, but also in proteinuric kidneys.

For therapeutic application of gene therapy in proteinuric renal disease, angiotensin-(1-7) [Ang(1-7)] is an attractive candidate. Ang(1-7) is known to have vasodilator properties in the renal vasculature through AT1 receptor antagonism, through Mas receptor stimulation and production of
nitric oxide (NO)\textsuperscript{20}. However, these data are opposed by other authors\textsuperscript{21,22}. Because the role of Ang(1-7) in the renal vasculature is not clear, in \textbf{Chapter 4} the effects of Ang(1-7) were compared in different experimental setups for evaluation of diverse sections of the renal vasculature. In all models studied, Ang(1-7) alone had no effect on the renal vasculature. \textit{In vitro}, Ang(1-7) antagonised angiotensin II (Ang II)-induced vasoconstriction of isolated renal arteries and isolated perfused kidneys. However, in the intact animal, Ang(1-7) was devoid of effects on Ang II-induced constriction of glomerular afferent and efferent arterioles measured under anaesthesia, and on Ang II-induced renal blood flow reduction in freely moving rats \textit{in vivo}. Thus, Ang(1-7) antagonises Ang II in renal vessels \textit{in vitro}, but does not have a major function in normal regulation of renal vascular function \textit{in vivo}. The Ang II-antagonising effects of Ang(1-7) observed \textit{in vitro} are most likely mediated by its AT\textsubscript{1} receptor antagonistic property. For this effect, high concentrations of Ang(1-7) were needed (µM range), in line with its low affinity for AT\textsubscript{1} receptor (µM range), compared to Mas (0.8 nM)\textsuperscript{23}. \textit{In vivo}, such high concentrations are unlikely to be achieved after intravenous infusion of Ang(1-7). Indeed, \textit{in vivo} the effects of Ang(1-7), in particular the effect on diuresis, are mediated by Mas\textsuperscript{24,25}. Exploration of the distribution and function of Mas receptors in the renal vasculature is therefore of pivotal importance, although their role in the regulation of renal vascular function \textit{in vivo} appears to be limited. Moreover, as put by Brunner and Gavras in an editorial comment to this work, “\textit{additional new in vitro data supporting the role of Ang(1–7) should not be considered sufficient to contradict the current in vivo results, unless some basic flaw in these studies has been overlooked. The challenge remains to present solid and convincing long-term data demonstrating a vasoactive effect of physiological concentrations of Ang(1–7) in vivo. To date, this evidence is not available}”\textsuperscript{26}.

Although Ang(1-7) has no important function in the physiological regulation of the vascular function of healthy kidneys, the role of Ang(1-7) in renal disease remains to be explored. Because adenovirus can efficiently transduce skeletal muscles, intramuscular injection of adenovirus is an elegant way to provide long-term exposure to a transgene protein after a single injection\textsuperscript{27}. Using this technique, the therapeutic potentials of gene therapy with an adenovirus harbouring an Ang(1-7)-expressing gene [Ad-IgPAng(1-7)] was investigated in the adriamycin model of proteinuric renal disease. Much to our surprise, not only Ad-IgPAng(1-7), but also control adenovirus (Ad-IgP) reduced proteinuria. Subsequently, we further investigated this antiproteinuric effect of recombinant adenovirus (\textbf{Chapter 5}). Intramuscular injection of adenovirus reduced proteinuria by 30-35% with a delay of 24-48 hours. This effect occurred irrespective of the transgene incorporated and was not associated with the application of anaesthesia, nor with a reduction in blood pressure, urine volume or body weight. In addition, renal function and histology and serum protein levels were stable after the injection of adenovirus. The effect was less prominent after repeated intramuscular injections. The antiproteinuric effect of adenovirus was independent of the dose / number of viral particles injected and more pronounced after intravenous injection. UV-irradiation of adenovirus, inactivating viral DNA, completely abolished the antiproteinuric response. Because of the delayed response, the desensitisation after repeated injection, the higher response after intravenous injection, the presence of an response irrespective of the incorporated
transgene, and the absence of response to viral particles without DNA, a mechanism involving an immune response to newly expressed viral proteins is the most plausible. As the antiproteinuric effect of adenovirus is substantial and reproducible, the therapeutic potentials of the mechanism underlying this effect should be explored further in proteinuric kidney disease.

Due to the unexpected observations after administration of control adenovirus, the capacity of Ang(1-7) as a therapeutic peptide in renal disease remains undefined. Therefore, Chapter 6 evaluates whether systemic infusion of Ang(1-7) reduces proteinuria and whether Ang(1-7) contributes to the renoprotective effects of the ACE inhibitor, lisinopril. In contrast to lisinopril, Ang(1-7) did not lower proteinuria and blood pressure. In addition, the antagonist of Ang(1-7), A779, was not able to inhibit the proteinuria and blood pressure-lowering effects of ACE inhibition. This shows that systemic Ang(1-7) plays no major role in the renoprotective effects of ACEi in adriamycin-induced nephrosis. These data are in sharp contradiction to earlier reports of Ang(1-7) contributing to the blood pressure lowering effect of RAAS inhibition. Recently, in a spontaneous hypertensive rat (SHR) model treated with an nitric oxide (NO)-synthase inhibitor, it was shown that Ang(1-7) reduced both blood pressure and proteinuria, and protected the kidney from histological damage. Two factors may be important for explaining these conflicting data. First, in models in which Ang(1-7) showed beneficial effects on the long term, this effect was consistently accompanied by a marked improvement of endothelial function, as shown for heart failure after myocardial infarction, in-stent restenosis, and SHR treated with an NO-synthase inhibitor. Ang(1-7) was ineffective in the early phase of a rat aortic banding model for renovascular hypertension (Loot, unpublished), but in this model endothelial function was still normal. In the adriamycin-model for proteinuric renal disease there is significant endothelial dysfunction, probably due to the direct exposure of toxic adriamycin to the vasculature. However, this endothelial dysfunction is unresponsive to both ACE inhibitor and Ang(1-7) therapy (van der Wouden, unpublished). Healthy endothelium of aorta relaxed in response to the endothelium-dependent vasodilator metacholine to approximately 50%. In adriamycin-nephrotic rats, the relaxation was only 27%, and could not be improved by lisinopril or Ang(1-7). Therefore, a first explanation may be that Ang(1-7) acts as a protective agent only in those diseases that display a marked, albeit improvable, endothelial dysfunction.

A second important factor in the (un)responsiveness to Ang(1-7) may be the prevailing state of the RAAS. The blood pressure-lowering effect of Ang(1-7) is observed in hypertensive rats and in rats consuming a low sodium diet, but is absent in normotensive animals. Similarly, the antidiuretic effect of Ang(1-7) was noticed in rats with high blood pressure (SHR and 2-kidney-1-clip model) and in sodium-depleted rats, but not in sodium-replete and normotensive rats. Together, these data indicate that activation of the RAAS is a conditional factor for the effects of Ang(1-7).

Consequently, in renovascular diseases with an activated RAAS, and (improvable) endothelial dysfunction, Ang(1-7) may have renoprotective properties. However, in normotensive proteinuric renal disease, Ang(1-7) is not renoprotective, and the beneficial response to ACE inhibitors is thought mainly
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due to the inhibition of Ang II formation. Future research should focus on the identification of conditions wherein the renoprotective capacities of Ang(1-7) emerge.

This thesis focuses on gene therapy and the optimisation of RAAS-inhibiting therapy in proteinuric kidney disease. Additional research has explored two other options which are included in appendices. The first appendix (Chapter 8) investigates a pharmacokinetic approach for optimisation of RAAS-inhibiting therapy. Previously, we observed a relative resistance to the antiproteinuric effect of ACE inhibition during the night when the drug was taken in the morning\textsuperscript{15}. Therefore, we studied whether alternative dosing of the long-acting ACE inhibitor, trandolapril, could overcome this nocturnal therapy resistance. In a prospective, randomised study, trandolapril was administered in the morning, in the evening, or bid to 14 nondiabetic proteinuric patients with a residual proteinuria of >1 gram on RAAS-inhibiting medication in a crossover fashion. However, residual proteinuria appeared to be equal during all dosing regimens. Therefore, the role of pharmacokinetics in the antiproteinuric effect of ACE inhibitors seems to be limited. This means for clinical purpose that once daily dosing of the long-acting ACE inhibitor, trandolapril, results in its optimal antiproteinuric effect.

Another option for optimisation of renoprotective therapy is explored in Chapter 9 and involves the interaction of the RAAS with the main profibrotic growth factor in kidney disease, transforming growth factor-ß (TGF-ß). TGF-ß is known to inhibit the response of vascular smooth muscle cells to Ang II. In this study, it is shown that TGF-ß reduces angiotensin type 1 (AT\textsubscript{1}) receptor levels, probably by reducing AT\textsubscript{1} receptor mRNA transcription. In conditions with increased TGF-ß, responses to Ang II may be importantly diminished by this interaction. This molecular mechanism, once identified, may be exploited to develop strategies to downregulate AT\textsubscript{1} receptors as an alternative RAAS-inhibiting therapy. Inhibition of the RAAS by AT\textsubscript{1} receptor antagonists\textsuperscript{36} and ACE-inhibitors\textsuperscript{37,38} features upregulation of AT\textsubscript{1} receptors in response to reduced receptor stimulation. The effectiveness of these drug interventions may be enhanced by strategies to downregulate AT\textsubscript{1} receptors.

Future directions

In conclusion, this thesis shows that gene therapy with adenoviral vectors is a feasible strategy in proteinuric kidney disease and, unexpectedly, offers a new lead to antiproteinuric therapy. The value of Ang(1-7) as a candidate gene appears to be limited in this perspective. Since RGD-modified adenovirus targets interstitial (myo)fibroblasts, intervention in the profibrotic role of these cells might halt the deterioration of renal function in CKD. For this purpose, gene therapy with smad7, which inhibits TGF-ß signal transduction, may be applied. Moreover, the unexpected antiproteinuric effect of adenovirus itself should be further investigated as it may offer a new strategy in the treatment of proteinuric kidney disease. Research should therefore focus on determination of the key antiproteinuric factor in this response and on its mechanism of action. In this perspective, experiments with helper-dependent adenovirus, lacking all viral genes, and plasmid-mediated gene transduction as well as characterisation of both structure and function of the glomerular basement membrane should be performed. Although, the role of Ang(1-7) in normotensive proteinuric renal disease seems to be limited, it may be worthwhile
to explore its therapeutic potential in haemodynamic models with profound endothelial dysfunction, such as the Fawn hooded hypertensive rats. Investigation of other strategies for optimisation of antiproteinuric therapy remains, however, of pivotal importance. Altered dosing of ACE inhibitors is not an effective strategy in this view, but downregulation of AT₁ receptors by TGF-ß may represent a new mechanism of action. However, the exact role of this interaction needs to be explored further.

This thesis does not solve the issue of progression towards end-stage renal disease in CKD, but it may contribute to our knowledge and understanding of resistance to the currently available therapies and open new perspectives for the optimisation of renoprotective therapy.

References
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