Summary and General discussion
Development of renal fibrosis is the final common pathway of chronic kidney disease (CKD), which ultimately leads to end-stage renal disease (ESRD) demanding life-saving therapies: renal dialysis or transplantation. Over time, the short- and long-term outcome of kidney transplantation have substantially improved, however, despite these improvements a large portion of grafts develop progressive dysfunction and fail. Loss of long-term kidney graft function resulting from progressive interstitial fibrosis (IF) is related to the number of rejection episodes earlier on (3). Recently another poorly understood phenomenon in kidney pathophysiology has come into focus: outgrowth of lymphatic vessels or ‘lymphangiogenesis’. This renal lymphangiogenesis has been observed in several kidney diseases ranging from renal transplantation to CKD, mostly associated and/or correlated with the extent of interstitial fibrosis (4). As such, renal fibrosis is an increasing global health problem (1, 2). Despite powerful renoprotective drugs, many patients still progress toward ESRD thereby imposing a great burden on the affected individual and on society (1).

Thus, there is a great need to develop new therapies to halt fibrogenesis and consequently arrest the progression toward ESRD. Expanding research in this field has explored new pathways and mechanisms involved in the pathogenesis of renal fibrosis and subsequently new therapies are under development. Most of the currently available therapeutic compounds eliminate rapidly from the body or poorly distribute into the kidney. In addition, these compounds may have systemic side effects. Therefore, in this thesis we tested the efficacy of various selective drug-targeting strategies aiming at reducing or preventing renal fibrosis using \textit{in vitro}, \textit{ex vivo}, and \textit{in vivo} models.

\textit{Chapter 1} provided a brief introduction to the topic and the scope of this thesis. \textit{Chapter 2} is a review on the renoprotective effects of protein kinase inhibitors and their specific delivery to target cells in the kidney, followed by future directions which may lead to novel specific pharmacological intervention strategies.

**Targeting proximal tubular epithelium in acute rejection**

As already mentioned, kidney transplantation has been recognized as the best therapeutic option for patients with ESRD (5). In spite of the progress in surgical techniques, the development of improved immunosuppressive agents, and a better understanding of immunologic phenomena, acute rejection remains a serious complication of kidney transplantation (6). Both cellular and humoral immune responses, and many different types of immune cells and cytokines, are involved in acute rejection, although the underlying pathophysiological mechanisms have not been fully elucidated. Activation of Rho-GTPases plays an important role in the regulation of actin cytoskeleton reorganization and inflammation (7, 8). Rho-associated coiled-coil protein kinase (ROCK) is one of the downstream effectors of Rho, and has been shown to play a role in inflammation and profibrotic processes in several models of renal damage (9, 10). For that reason, in \textit{Chapter 3}, we tested the efficacy of targeted delivery of a Rho kinase inhibitor in a rat acute renal allograft rejection model. Isografts were used as controls. As acute rejection is characterized by proximal tubular damage, we aimed to deliver a Rho kinase inhibitor Y27632 to this tubular compartment. A lysozyme-ULS-based drug targeting strategy was used to deliver
Y27632 to renal proximal tubular epithelial cells. Our study demonstrates that local activation of tubular Rho kinase plays an important role in macrophage chemoattraction into the renal tubulointerstitium. The pathophysiologic role of macrophage accumulation in acute allograft rejection is increasingly recognized (11). Besides their fundamental role in tissue remodeling during embryonic development and their immunologic role in host defense, macrophages also play a role in acquired kidney disease, and alloimmunity against renal allografts (12). Thereby, the reduction of the procollagen-1α1 gene in response to renal Rho kinase inhibition may be the consequence of reduced renal macrophage accumulation, although the Rho kinase pathway may also directly modulate collagen-1α gene expression (13, 14). We found a dose-dependent reduction of IL-β-induced MCP-1 expression by the Rho kinase inhibitor (Y27632) in cultured rat tubular epithelial cells, which might explain reduced accumulation of macrophages in our in vivo model. However, we could not demonstrate a reduction of total kidney MCP-1 mRNA expression by Y27632-lysozyme in our in vivo experiment. The reason could be that we did not specifically measure tubular epithelial cells MCP-1 expression in vivo, but we checked the whole kidney MCP-1 mRNA expression. Beside the effect on macrophage accumulation, Y27632-lysozyme also reduced the number of renal lymph vessels. In renal research, the exit route of recruited inflammatory cells, i.e. the lymphatic vessels, have recently received specific attention. During inflammatory injury, new routes are created by de novo formation of lymphatic vessels, a process referred to as lymphangiogenesis. These newly-formed lymphatic vessels help to cope with the increase in interstitial fluid related to inflammation. Although the current study does not provide a mechanistic explanation, we propose that the lower macrophage infiltration in the Y27632-lysozyme-treated group as compared to vehicle is involved in the reduction of lymphangiogenesis. Macrophages may play a major role in lymphangiogenesis, not only by producing high levels of chemokines and lymphangiogenic factors (15), but also by incorporating into the lymphatic vessel wall (16). Therefore, reduction of lymph vessel numbers in treated animals compared to controls, could be secondary to decreased macrophage influx. Based on the results obtained from this study we envision that renal delivery of Rho kinase inhibitors could be a valuable future treatment in renal transplantation. Several inhibitors of Rho kinase (in particular fasudil and Y27632) have been extensively used to evaluate the importance of ROCK in disease conditions. Fasudil is used in Japan to treat cerebral vasospasm after focal cerebral ischemia or aneurysmal subarachnoid hemorrhage (17). However, both inhibitors have a moderate specificity and potency, and are short-acting in vivo which limit their clinical potential. Here, we described the short-term effects of Y27632 in an acute model of kidney injury, however, it would of interest to next 1) evaluate whether Rho kinase inhibition confers protective effects in a model of more advanced renal disease such as diabetic nephropathy or hypertension, 2) evaluate novel Rho kinase inhibitors such as SAR407899 using targeting strategies, and 3) examine the efficacy of Rho kinase inhibitors in combination with RAS blockers. RAS inhibitors are likely to have an important impact on renoprotection in diabetes. Studies have suggested possible synergy between high glucose- and angiotensin II (AngII)-induced RhoA/ROCK signaling in mesangial cells, attenuation of glomerular microvascular actions
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of AngII by Rho kinase inhibition, and beneficial effects of this inhibition on AngII- and aldosterone-induced renal injury (18-21). Therefore, combination therapy using targeted delivery of ROCK inhibitor and an angiotensin II receptor 1 blocker (ARB) could enhance the therapeutic effects. Another interesting issue is the link between the Rho/ROCK pathway and klotho expression. It was revealed that RhoA/ROCK signaling pathway activation is closely associated with the expression of klotho (22). Klotho, a gene that has been identified as an anti-aging factor, is predominantly expressed in the choroid plexus of the brain and distal convoluted tubules of the kidney in healthy individuals. A significant decline in klotho gene and protein expression has been reported in the kidneys of diabetic rats (23). Klotho overexpression ameliorated renal hypertrophy and fibrosis, and is considered to be a safe therapy since overexpression of klotho extends the lifespan of mice (24). However, further studies are needed to reveal and elucidate the exact role of klotho on the activity of ROCK. This may offer a novel approach for developing new ROCK inhibitors.

Targeting interstitial myofibroblasts in renal fibrosis

We then moved forward to explore specific drug delivery to myofibroblasts as ‘main extracellular matrix producing cells’. Although data on the reversibility of fibrotic processes in the kidney are scarce, few studies indicate that resolution of renal fibrosis does exist (25-27). The clearance of activated fibroblasts is particularly important in order to provide therapeutic strategies to inhibit the progression of tubulointerstitial fibrosis. As described in Chapter 4, interferon gamma (IFNγ), a cytokine with anti-fibrotic effects, was delivered to platelet-derived growth factor β (PDGFRβ)-expressing myofibroblasts in mouse kidneys subjected to unilateral ureter obstruction (UUO). To identify the efficacy of our strategy, we examined α-SMA expression both on mRNA and protein level. We indeed observed a clear inhibitory effect of PPB-PEG-IFNγ on myofibroblast activation in vitro and in vivo. Compared to free IFNγ, targeted PPB-PEG-IFNγ showed enhanced anti-fibrotic effects (reduced fibronectin and collagen I expression), which stresses the added value of cell-specific targeting. Accumulation of myofibroblasts is also associated with loss of epithelial integrity and tissue architectural distortion. We showed that targeted IFNγ improved tubular morphology, which might be explained by interaction and communication between these two cell types. It has been shown that secreted fibroblast-derived miRNAs induce tubular cell apoptosis in obstructive kidneys which contributes to tubular atrophy (28). We also observed decreased T cell infiltration and reduced lymphangiogenesis in treated kidneys. Importantly, targeted IFNγ reduced brain MHC class II expression (a systemic side effect of IFNγ in this model) when compared with free IFNγ. However, one of the remaining questions is obviously the mechanism by which targeted IFNγ attenuated fibrosis. Biologic effects of IFNγ take place via the nuclear signaling sequence (NLS), which is present in its C terminus region (29, 30). This region is able to modulate IFNγ-responsive genes through activation of the JAK/STAT pathway (Janus kinase/signal transducers and activators of transcription signaling pathway) (31, 32). PEG-PPB-IFNγ was previously shown to activate STAT1 (33). We therefore propose that PEG-PPB-IFNγ is taken up via PDGFRβ, and the internalized construct next releases IFNγ or its metabolite intracellularly, which then binds to the intracellular
part of IFNγ receptor 1 (IFNγR1). IFNγ-R1 indeed has the JAK1 and STAT1-binding site on the intracellular part. IFNγ containing the NLS was capable of binding to IFNγR1 and initiate a cascade of events, which are required for nuclear import of STAT1 and generation of anti-fibrotic activity. However, this proposed mode of action of IFNγ needs to be further explored. In general, our findings demonstrate that specific targeting of IFNγ to PDGFRβ-expressing myofibroblasts attenuates renal fibrosis and reduces systemic adverse effects, which holds potential as therapeutic strategy in renal fibrosis. Given our data it is of interest to test the efficacy of this construct in chronic kidney disease models and see if renal fibrosis is reversible. Apart from its use in models of fibrosis, we propose that this construct can also be a therapeutic candidate for HIV-1-associated nephropathy (HIVAN) since renal interstitial scarring is a predominant component of HIV-associated nephropathy (34).

Despite the beneficial effects described in Chapter 4, the presence of extracellular IFNγ receptor binding site in PPB-PEG-IFNγ might still induce some adverse (pro-inflammatory) effects during long-term administration. To address this, in Chapter 5, we evaluated the anti-fibrotic potency of targeted IFNγ peptidomimetic (mimγ) which lacks the IFNγR recognition part while retaining IFNγ-mediated anti-fibrotic functions in order to further reduce systemic side effects. We observed severe fibrosis in UUO mouse kidneys which is characterized by markedly increased renal α-SMA, fibronectin, and collagens I and III expression. mimγ-BiPPB significantly reduced fibrotic marker expression both on particularly the protein level. Attenuated fibrosis was associated with reduced renal lymphangiogenesis. Compared with non-targeted full length IFNγ, mimγ-BiPPB decreased IFNγ-related side effects manifested by reduced brain MHC II expression, lowered plasma triglyceride levels and improved weight gain after induction of UUO. These results indicate that specific targeting of mimγ-BiPPB to PDGFRβ-expressing myofibroblasts attenuates renal fibrosis and prevents IFNγ-induced systemic adverse effects. Although the mechanisms by which targeted IFNγ attenuated fibrosis need to be investigated, however, we assume that the biologic effects of IFNγ take place via the NLS which can modulate IFNγ-responsive genes through activation of the JAK/STAT pathway as already discussed above.

To broaden the targeted drug delivery strategy, we envision the possibility of targeted combination therapy to both proximal tubular epithelial cells and myofibroblasts at the same time. From a drug targeting point-of-view, both proximal tubular cells and myofibroblasts are attractive target sites (35, 36). Via administration of IFNγ-LZM and IFNγ-PPB we might be able to block the STAT pathway in both cell types and therefore improve the therapeutic index.

**Precision-cut kidney slices to study renal fibrogenesis**

In order to develop novel anti-fibrotic therapies detailed knowledge of the underlying pathophysiology is necessary as well as the availability of relevant model systems that enables rapid screening of anti-fibrotic effects of compounds. Precision-cut tissue slices (PCTS), a three-dimensional multicellular environment, is a powerful tool to provide insight into mechanisms of organ injury (37-41), although its usage as a model for renal fibrosis is not investigated yet. Therefore, in Chapter 6 we developed and tested a mouse kidney ex
vivo slice model to study renal fibrosis. Precision cut kidney slices (PCKS) is a multicellular system in which cell-cell and cell-extracellular interactions are maintained. Our data revealed the applicability of PCKS as an ex vivo model to study renal fibrogenesis, as well as a model to test anti-fibrotic effects of IFNγ and targeted IFNγ, aiming at reducing renal fibrosis. Specifically, the results demonstrated that incubation of mouse PCKS (mPCKS) with TGFβ1 resulted in the upregulation of fibronectin, collagen I, and α-SMA expression which indicates that mPCKS represent a useful model to study the onset of fibrosis. Preserved expression of α-SMA suggests that fibroblasts remained active during ex vivo culture. Intervention with free IFNγ and the targeted PPB-PEG-IFNγ conjugate in mPCKS clearly dampened TGFβ1-induced expression of fibronectin, collagen I and collagen III, indicating the anti-fibrotic potential of both free and targeted IFNγ in this model. In summary, from the results of this work we conclude that the observed anti-fibrotic effects of free IFNγ and PPB-PEG-IFNγ in vivo can be successfully reproduced using mPCKS. These results point out that this ex vivo model is a valuable tool for preclinical studies to test the efficacy of potential new anti-fibrotic drugs in a multicellular, profibrotic milieu. Importantly, it provides the opportunity to study these processes not only in rodent-derived kidney tissue, but also in (fibrotic) human kidney tissues. Additionally, the use of this model will contribute to the reduction, refinement, and potential replacement of animal experiments. Testing different targeted constructs in both healthy and fibrotic slices of human kidneys will be the future experiments in order to develop clinically available anti-fibrotic therapy.

**Lymphangiogenesis in proteinuric renal disease**

On the last part of this thesis we aim to explore the mechanism of de novo formation of lymphatic vessels as this is a common finding during many renal fibrotic diseases. Injured tubular cells and inflammatory cells secrete several mediators and growth factors that promote lymphangiogenesis (4). In Chapters 3, 4 and 5 we observed reduced lymphangiogenesis after therapeutic intervention in models of acute rejection and renal fibrosis. However, based on these data we were not able to conclude whether lymphangiogenesis is a response secondary to the development of renal injury and fibrosis, or whether it is actually a driving force and promoting factor. Therefore, in Chapter 7 we investigated the temporal relationship between development of fibrosis, inflammation and lymphangiogenesis in a chronic progressive kidney disease model. We showed that the formation of renal interstitial lymph vessels occurs after established proteinuria but prior to collagen deposition, fibrosis, and macrophage influx. This suggests that renal lymphatic vessel activation and lymphangiogenesis is causally involved in the development of renal fibrosis and inflammation. This proposes that renal lymphangiogenesis, at least in some disease conditions, can serve as potential therapeutic target to dampen inflammatory and fibrotic processes involved in tubulointerstitial remodelling. Nevertheless, lymphangiogenesis can likely be seen as a double-edged sword in kidney diseases. From one hand, renal lymphangiogenesis can promote the increased efflux of lymph and inflammatory cells, thereby exerting a protective influence. On the other hand, the increased number of lymphatic vessels might stimulate acquired immune responses against the endogenous
kidney or renal allografts, which might be detrimental in the long run. The important question of whether lymphangiogenesis is good or bad in the long run remains a matter of debate. Therefore, improved understanding of the detailed mechanisms involved in renal lymphatic functions and remodelling are needed and require future research which might provide possible therapeutic interventions for curative or preventive approaches in renal disease and graft survival.

CONCLUSIONS

Focus of this thesis is on the intracellular delivery of Rho kinase inhibitor to renal proximal tubular cells and IFNγ to myofibroblasts, the development of a new technique to study renal fibrosis ex vivo, and finally the role of lymphangiogenesis and its temporal relation to inflammatory and fibrotic responses in the development of progressive renal disease.

Given that many pathways of tissue injury are shared between disease entities and organ physiology, non-selective therapeutic targeting of these pathways are expected to have undesired side effects. Hence, selective targeted delivery may identify a strategy that is effective across a spectrum of renal and non-renal conditions. In addition, AKI and CKD are frequent clinical conditions and applying this approach may allow repurposing of drugs already studied or used in the context of other diseases.

In general, the high blood flow to the kidneys, the renal excretion of metabolites and many drugs, as well as the complexity of transporters of tubular cells, provide the possibility to design kidney targeted drugs either de novo or through modification of previously developed ones. The site- and time-specific drug targeting remains a major challenge. Thus, targeting and controlled release of drugs are areas of intensive research. Drug delivery to the specific cells in the kidney not only offers the possibility to improve the therapeutic indices of drugs that give severe side effects, but also to gain more insight into the signaling cascades that are responsible for the disease progression, such as profibrotic actions during tubulointerstitial fibrosis.

In summary, in this study, we demonstrate the benefit of cell specific targeted delivery to attenuate graft rejection, inflammation and fibrosis in the animal models of kidney disease. We provide a novel strategy of drug delivery to proximal tubular cells by using megalin receptor, and to myofibroblasts by directing anti-fibrotic cytokine to PDGFR. These strategies increase drug therapeutic potential due to enhanced efficacy and reduced off-target side effects. We believe cell-specific drug delivery is a promising approach to effectively halt renal fibrosis. Moreover, the ex vivo model which we described here is a novel tool to test the pathophysiology of early onset as well as end-stage of fibrosis and to screen the efficacy of anti-fibrotic drugs ex vivo in a multicellular and pro-fibrotic milieu. Major advantage of the slice model is that the data from our animal study can be directly extrapolated to human situation by using (fibrotic) human kidney tissue. Importantly, tissue slices is an alternative or 3R methods which give insight into mechanisms of disease processes and is designed to replace, reduce, and refine animal experiments.
REFERENCES


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