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Molecular markers in renal transplant biopsies

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Chapter 1

General Introduction





General introduction

The development of chronic allograft nephropathy (CAN) is the primary cause of late allograft dysfunction in kidney transplants ^{1,2}. CAN is characterized by tubulointerstitial fibrosis and tubular atrophy. Renal interstitial fibrosis is the result of overt accumulation of extracellular matrix (ECM) components. The Banff classification is used to score the extent of CAN in renal transplant biopsies ³.

This thesis focuses on the identification of molecular markers in renal transplant biopsies that are predictive of late graft function. Potential molecular markers were investigated at the mRNA level with quantitative polymerase chain reaction (Q-PCR) and microarray analysis, and at the protein level with immunohistochemistry. Molecular markers in kidney transplant biopsies have four prime applications. First, they can aid in the diagnosis of the disease to facilitate early treatment. Second, they can elucidate the pathogenesis of disease and provide a basis for new therapeutic strategies designed to target allograft dysfunction. Third, they serve as a prognostic tool to determine disease severity and guide treatment choices. Fourth, they can be employed in monitoring the effects of a therapeutic intervention and inform dose adjustments.

In this general introduction, an elaboration on kidney transplantation will be provided. Different mechanisms that lead to the development of CAN will be discussed, focusing on ECM accumulation in the tubulointerstitial compartment as the hallmark of CAN. The general introduction will be concluded with a summary of the aims of the individual chapters of this thesis.

Kidney transplantation

The first successful renal transplantation was performed in Boston in 1955 by Joseph Murray and Hartwell Harrison ⁴. Over the last three decades the technique has improved and graft survival has been extended substantially ⁵. Since the introduction of potent immunosuppression by calcineurin inhibitors in the early 1980's, the first year graft survival rate has increased to 90–95% ^{6,7}. The primary cause of graft failure in the first year is the occurrence of acute rejection (AR). Long-term graft survival has also increased, but to a lesser extent ⁸. At ten years post transplantation approximately 40% of all allografts fail; this is mainly due to the development of chronic allograft nephropathy (CAN) ^{1,2}.

Chronic allograft nephropathy

CAN is defined by the histopathological features of tubulointerstitial fibrosis and tubular atrophy. The presence of CAN in the renal allograft usually culminates with a decrease in renal function or renal failure. The histological alterations associated with CAN result from complex interactions between immunological and non-immunological conditions including: (sub)clinical AR, nephrotoxicity as a result of chronic exposure to immunosuppressive agents ⁹, and pre-existing morphological abnormalities from donor derived vascular disease. Both chronic rejection activity and chronic calcineurin inhibitor administration can lead to the development of CAN ¹⁰. However, it should be noted that CAN is not a generic term and should not be misused in determining a diagnosis ¹¹.

Acute rejection

The primary risk factor for the development of CAN is the occurrence and quality of AR episodes, where the important parameters include the type, time after transplantation, quality, and frequency¹²⁻¹⁴. In addition, the functional response to therapy is important in distinguishing between acute rejection episodes that will or will not impact graft survival¹⁵. The type of acute rejection may be vascular or interstitial; the former has a less favorable long-term prognosis¹⁶. For example, diffuse C4d deposition in the peritubular capillaries during an AR indicates a humoral AR episode, and is associated with an inferior long-term prognosis¹⁷. A clinical AR episode that occurs within the first 3 months after transplantation and is adequately treated is less detrimental for long-term survival than an AR episode after this period^{14,18}. In a prospective study, the amount of human leukocyte antigen-DR (HLA-DR) mismatches predicted the presence of subclinical acute rejection in protocol biopsies obtained at 3 months, indicating a donor-specific immune response¹⁹. In protocol biopsies obtained 6 months after transplantation, Scholten *et al.*²⁰ reported that over 30% showed signs of acute rejection according to the Banff classification³. In addition, a subclinical (untreated) rejection episode was not associated with the deterioration of renal function over time in the intermediate term; this indicated that asymptomatic infiltrate may play an adaptive, rather than destructive, role. In a larger prospective randomized study of protocol biopsies, the results presented by both Naesens and Scholten should be investigated and verified simultaneously to assess their value. The frequency of AR episodes affects long-term graft survival; transplant recipients that experienced only one AR episode had a better graft prognosis than those with more than one episode¹³.

Calcineurin inhibitor toxicity

With the introduction of calcineurin inhibitors in the early 1980's, there has been a reduction in AR episodes⁵. The calcineurin inhibitors, Cyclosporine A (CsA) and Tacrolimus (Tac) prevent T-cell activation through inhibition of interleukin-2 (IL-2) transcription. However, in initial investigations into the use of calcineurin inhibitors for immunosuppression in kidney transplantations, Calne *et al.* observed that calcineurin inhibitors exerted nephrotoxic side effects²¹. Other studies have suggested that the development of CAN may in part be attributed to the use of calcineurin inhibitors^{9,22}. Indeed, a study using protocol biopsies showed that at 10 years virtually all allografts showed pathological changes consistent with CsA toxicity and exacerbated CAN²³. Animal studies showed that calcineurin inhibitors caused increased mRNA expression of transforming growth factor- β (TGF- β) in the kidney²⁴⁻²⁶. Furthermore, nephrotoxic lesions in the kidney were prevented through the use of anti-TGF- β antibodies²⁴. Calcineurin inhibitors also induce extracellular matrix (ECM) protein expression; this may be mediated by the increased levels of TGF- β ²⁷⁻²⁹. In addition, CsA has been shown to target the promoter fragment of collagen $\alpha 1$ (III)³⁰; this direct induction may also contribute to the overt accumulation of ECM.

Many previously published studies involving patients with renal transplants have shown discordant results with respect to the fibrotic potential of the calcineurin inhibitors CsA and Tac³¹⁻³⁹. This discrepancy might be due to differences in patient inclusion criteria or the materials and methods used. Some studies included biopsies taken for diagnostic purposes or protocol biopsies from patients that had an AR episode just before or at the time of biopsy. Including biopsies with AR and those taken on the basis of clinical indications may produce biased results in TGF- β expression studies. Furthermore, differences with respect to the renal compartment studied, either isolated glomeruli or whole cortex, could give rise to variations in the results³⁴. Finally, mRNA expression studies that investigated the fibrogenic impact of different immunosuppressive regimens did not combine mRNA and protein analyses and thus did not elucidate whether the increased mRNA expression levels were accompanied by increases in protein expression^{31,34}.

Protocol biopsies

Surveillance or protocol biopsies, obtained within the first year after transplantation, might facilitate the detection of AR or CAN at an early stage and allow earlier intervention⁴⁰⁻⁴². Protocol biopsies have not yet been widely implemented in routine care because the beneficial value remains controversial. The potential early detection of subclinical rejection, CsA nephrotoxicity, BK virus, and CAN argue for the use of routine protocol biopsies. However, the benefit of early treatment remains unknown and the lack of proof that protocol biopsies lead to improvements in long-term graft survival argues against routine protocol biopsies⁴¹. Moreover, previous studies have shown that for monitoring the progression of CAN, it is sufficient to perform one donor biopsy (t0) and one recipient protocol biopsy during the first year after transplantation^{43,44}.

Mechanisms that give rise to interstitial fibrosis

The tubulointerstitium (TI) is a matrix of fibrous structural proteins, adhesive glycoproteins, proteoglycans, and hyaluronic acid⁴⁵. Fibroblast-like and lipid-laden interstitial cells constantly secrete extracellular matrix proteins that form the TI matrix. Matrix metalloproteases (MMPs) are responsible for ECM degradation and include the interstitial collagenases (MMP-1, MMP-2, MMP-8, and MMP-13), stromelysins, gelatinases (MMP-2 and MMP-9), and elastases⁴⁶. Thus, the normal equilibrium of ECM is a delicate balance of synthesis and degradation. Interstitial fibrosis develops when this balance is disrupted by increases in transcription, translation, local production, and deposition of ECM proteins and/or a decrease in protein degradation. The resulting accumulation of interstitial matrix molecules gives rise to interstitial fibrosis (Fig. 1).

The two-faced cytokine, TGF- β

TGF- β is a multifunctional cytokine expressed in three isoforms with nearly identical biological properties. TGF- β 1 has been shown to induce excessive ECM protein deposition⁴⁷. TGF- β receptors at the cell surface initiate signal transduction to the nucleus through the phosphorylation of Smad 2 and Smad

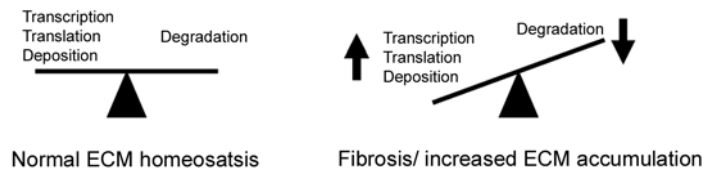


Figure 1. Schematic drawing of extracellular matrix (ECM) homeostasis. ECM homeostasis is a delicate balance between synthesis and degradation. Disruption of this balance causes an excessive accumulation of interstitial matrix molecules. Adapted from Eddy *et al.* ⁶².

³ ^{48;49}. TGF- β signaling is inhibited by a negative feedback mechanism through the activation of Smad 7 ⁵⁰. *In vivo* and *in vitro* studies have shown that TGF- β can enhance the expression of many ECM molecules, including collagen I, III, and IV, fibronectin, laminin, and decorin ^{29;51;52}. Furthermore, TGF- β can inhibit matrix degradation by reducing the activity of MMPs and increasing the activity of tissue inhibitors of metalloproteases (TIMPs) ^{52;53}.

In addition to its fibrogenic effect, TGF- β also has an anti-inflammatory effect ⁵⁴. Mice with an inactivated TGF- β gene die within four weeks due to excessive inflammation in multiple organs ⁵⁵. In contrast, transgenic mice that overexpress TGF- β exhibit a reduction in fibrosis due to the anti-inflammatory effects of TGF- β ⁵⁶. Furthermore, when mice with cardiac transplants were given plasmid DNA that encoded TGF- β the allograft survival rate was prolonged ^{57;58}. This result is consistent with studies that showed TGF- β can deactivate macrophages and inhibit the activation of cytotoxic T-lymphocytes ^{59;60}. In accordance with this, Eikmans *et al.* found increased TGF- β mRNA expression levels in AR biopsies from patients that did not progress to chronic allograft failure over time ⁶¹.

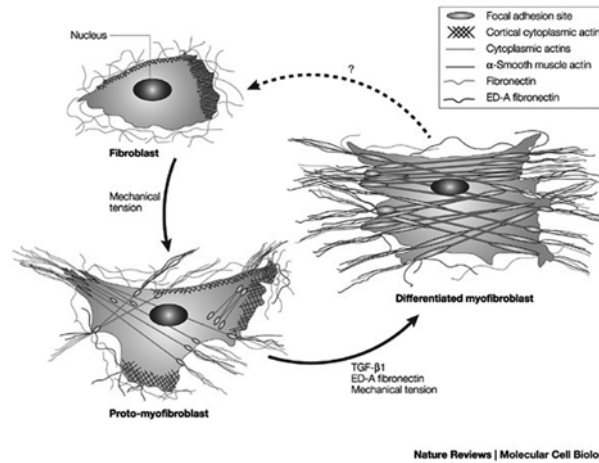
Myfibroblasts contribute to fibrogenesis

The maintenance and turnover of ECM in organs is largely controlled by residing fibroblasts. This control is especially important in renal transplantation. In renal injury, fibroblasts become activated and undergo a phenotypic and functional conversion into myfibroblasts. Once converted, they rapidly proliferate and produce excessive ECM molecules ⁶²⁻⁶⁴. Myfibroblasts are active during normal wound healing, but once epithelial restoration is complete, myfibroblasts undergo massive apoptosis ⁶⁵. However, under conditions that favor fibrosis, this wave of apoptosis is virtually absent. Moreover, the number of myfibroblasts in the tubulointerstitium is directly correlated with the degree of interstitial fibrosis ⁶⁶.

Schürch *et al.* defined myfibroblasts as cells that possess three essential morphologic elements:

- 1) stress fibers,
- 2) well-developed cell-to-stroma attachment sites, and
- 3) intercellular intermediate and gap junctions ⁶⁷.

The conversion of a fibroblast into a myfibroblast can be described in a two stage model (Fig. 2) ⁶⁵. First, due to changes in the microenvironment, for example tissue remodeling or initial wound healing, fibroblasts evolve into proto-myfibroblasts that express vimentin and cytoplasmic (β and γ) actins.



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Figure 2. A two-stage model of myofibroblast differentiation. Fibroblasts evolve into proto-myofibroblasts due to changes in the microenvironment. Later, in the presence of growth factors and ECM molecules, the proto-myofibroblast differentiates into a myofibroblast. Printed with permission from Macmillan Publishers Ltd: Nat Rev Mol Cell Biol. Tomasek JJ, et al. Myofibroblasts and mechano-regulation of connective tissue remodelling. Nat Rev Mol Cell Biol 2002; 3(5):349–363, copyright 2002⁶⁴.

Second, in the presence of growth factors and ECM molecules, the proto-myofibroblasts become differentiated myofibroblasts and express variable amounts of desmin and alpha-smooth muscle actin (α -SMA)^{68;69}. The differentiated myofibroblast is responsible for contraction in wound healing, but is also present in most fibrocontractive diseases. TGF- β is the main activator of the proto-myofibroblast transition into a differentiated myofibroblast⁶⁵.

Epithelial to mesenchymal transition

Interstitial fibrosis is characterized by the presence of myofibroblasts; however, their origin is subject to debate. Most agree that myofibroblasts are recruited from bone marrow and arise from proliferation of local myofibroblasts. However, recent evidence suggested that about 36% of new fibroblasts were formed from tubular epithelial cells (TECs) through a mechanism called epithelial to mesenchymal transition (EMT)^{70–72}. In response to injury, renal epithelial cells can revert to an embryonic, metanephric/mesenchymal phenotype (Fig. 3). Then, possibly due to pathological manipulation of the cellular environment, these cells may undergo a further transition into a mesenchymal phenotype. In this state, cells can migrate through the tubular basement membrane into the ECM and become involved in the production of ECM proteins⁷³.

EMT-associated molecules

Through EMT, TECs can become ECM producing cells. However, both *in vitro*, and *in vivo*, TECs exhibited mRNA transcription of collagen genes and contributed to the accumulation of ECM molecules in the apparent absence of EMT^{74–76}. This indicates that both myofibroblasts and tubular cells may contribute

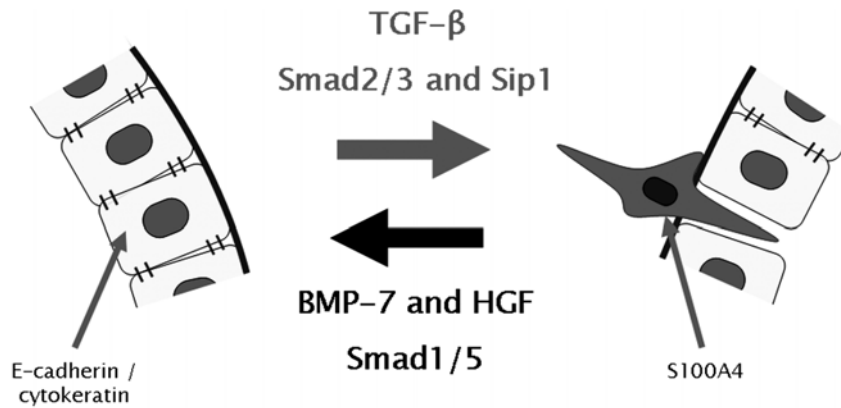


Figure 3. Schematic drawing of epithelial to mesenchymal transition (EMT). During EMT the characteristic epithelial phenotype is lost and a mesenchymal phenotype is acquired. The mesenchymal phenotype is identified by S100A4 expression. TGF- β is the prime inducer of EMT through a Smad 2/3 and Sip1 pathway. This process can be reversed by BMP-7 through a Smad 1/5 dependent pathway and HGF.

to the process of interstitial fibrosis. Nevertheless, *in vivo*, it has been shown that TECs can lose their characteristic cytokeratin expression and gain a mesenchymal phenotype; this supports the hypothesis that TECs can undergo EMT^{77,78}.

EMT involves shutting down transcription of epithelial-specific genes and inducing transcription of mesenchymal-specific genes. Specific markers for EMT include a reduction of E-cadherin (TEC specific) and induction of α -SMA (myofibroblast specific) expression. *In vitro* studies have shown that adding TGF- β to TECs induced collagen α 1(I), α -SMA, and fibroblast specific protein 1 (FSP-1) expression and reduced E-cadherin expression, indicating that TGF- β may be a mediator of EMT^{71,79,80}. Moreover, TGF- β receptors are rapidly up-regulated in TECs of diseased kidneys, suggesting TGF- β may target TECs in injured states⁸¹. TGF- β can induce the transcriptional factors, Smad-interacting protein 1 (SIP1) and Snail; this represses E-cadherin expression and may contribute to EMT⁸²⁻⁸⁶. Finally, TGF- β modulation occurred in a Smad 3-dependent manner⁸⁷⁻⁸⁹. This is notable because Smad 3 knockout mice exhibited reduced EMT, monocyte influx, and collagen accumulation in a unilateral ureteral obstruction (UO) model compared to wild-type littermates⁸⁷.

EMT in human renal biopsies

Previous studies in human renal biopsies have investigated the presence of EMT-related markers at the protein level^{75,90-92}. These studies showed that human TECs can also undergo EMT and thus contribute to interstitial fibrosis. The number of TECs that were positive for mesenchymal markers correlated with a decrease in renal function^{75,92}, and with the progression of renal disease⁹⁰. Furthermore, in protocol biopsies of patients with stable renal allograft function, there were almost no TECs that exhibited a mesenchymal phenotype⁹².

Protective and regenerative factors in EMT

Bone morphogenetic protein-7 (BMP-7), a member of the TGF- β superfamily, is the natural antagonist of TGF- β . BMP-7 inhibits TGF- β through the induction of Smad 7 and regulates its own activity via a negative feedback mechanism involving the production of Smad 6⁵⁰. BMP-7, also known as osteogenic protein 1, is crucial during embryonic kidney development, when it is responsible for mesenchymal to epithelial transition (MET) that induces branching morphogenesis^{93;94}. The biological effects of BMP-7 are mediated through the phosphorylation of Smad proteins 1, 5, and 8^{48;49}. It has been proposed that BMP-7 expression may counteract and reverse chronic renal injury and induce tissue regeneration by inducing MET⁹⁵. In animal models, BMP-7 expression inhibited progression of fibrosis, and also counteracted EMT that was induced by TGF- β ^{96;97}.

The cytokine hepatocyte growth factor (HGF) was recently shown to antagonize the fibrogenic actions of TGF- β ⁹⁸⁻¹⁰⁰. In animal models, HGF prevented the onset and progression of chronic kidney disease¹⁰¹. Liu *et al.* showed in cultured proximal TECs that HGF inhibited tubular EMT and maintained the epithelial phenotype^{102;103}. HGF also promoted ECM degradation through the induction of MMPs and reduction of TIMP expression^{104;105}. However, the prognostic relevance of EMT-related molecules in renal transplants still remains to be determined.

Methods of investigating molecular markers in kidney tissue

Gene expression analysis can facilitate the investigation of the development of CAN and the influences of AR, calcineurin toxicity, and EMT^{61;106}. The Q-PCR technique enables the analysis of small quantities of mRNA obtained from renal allograft biopsies. In addition, microarray analysis enables the analysis of mRNA expression profiles for thousands of genes within a small tissue sample in a relatively short time period. This method can give valuable insight into the underlying molecular pathways and pathophysiology of the disease. However, without a distinct research hypothesis, a microarray analysis is a shot in the dark, and could lead to more uncertainty than clarification. After microarray analysis, it is vital to keep in mind that the results require validation in an independent group of individuals, using alternate methods like Q-PCR. Later, *in situ* hybridization on paraffin embedded tissue sections is performed to determine the site of mRNA expression. Immunohistochemical stains are performed to determine the site of protein deposition in tissue, keeping in mind that the protein is not necessarily located at the site of mRNA transcription. Finally, computer image analysis is performed to quantify protein deposition in the slides and assess relationships between protein deposition and renal allograft outcomes.

Clinical applications of molecular markers

There are four prime applications for molecular markers. First, investigation of molecular markers in renal tissue might be of diagnostic value. For example, it is currently difficult to distinguish between calcineurin inhibitor toxicity and chronic rejection solely on the basis of their histological and clinical differences. Koop *et al.* used mRNA expression analysis to show that the expression levels

of laminin $\beta 2$ and TGF- β could be used to discriminate between the two disease pathologies with high specificity and sensitivity¹⁰⁷. Second, molecular markers could be used to monitor the effects of therapy and thus aid in the prevention of tissue damage due to toxicity.

Third, the assessment of mRNA expression levels and quantification of protein deposition may identify early predictive molecular markers. These could be useful in the quest for therapies that improve allograft survival over time. Early TGF- β , collagen $\alpha 1$ (IV), and decorin expression levels were shown to be significantly increased in patients that had stable graft function after 5 years compared to those who had graft failure at 5 years⁶¹.

Finally, assessments of gene expression in transplant biopsies could identify pathways that are associated with graft survival and rejection. DNA microarray analysis of acute rejection biopsies showed that dense clusters of B-cells were strongly associated with severe graft rejection¹⁰⁸. This knowledge may provide insight into disease mechanisms and aid the design of novel pharmaceutical agents that target key molecules in disease pathways. This approach may lead to the discovery of novel drugs that increase long-term graft survival.

Scope of this thesis

The central theme of this thesis is that mRNA expression and protein deposition assessments can be used to predict renal allograft outcomes and monitor the (negative) effects of therapeutic interventions. Moreover, the identification of molecular markers might provide insight into new therapeutic agents that improve graft function. First, in order to investigate molecular markers, it is necessary to optimize mRNA extraction methods to improve the quantity and quality of mRNA for analysis. This could lead to improved methods that allow the implementation of mRNA studies in routine diagnostics (Chapter 2). Next, these methods were used to address the conundrum of immunosuppressive therapy: on one hand it appears to increase graft survival, and on the other hand it contributes to the development of chronic renal transplant dysfunction. In Chapter 3 we investigated the difference in fibrogenic effects between the widely used calcineurin inhibitors CsA and Tac. Both mRNA and protein expression of fibrogenic markers were evaluated in protocol biopsies obtained from patients that were randomized to receive tailored regimens of the currently available calcineurin inhibitors.

The primary risk factor in the development of CAN is the occurrence of acute rejection episodes. The goal of Chapter 4 was to identify and validate genes expressed during acute rejection and assess correlations to the progression to CAN.

EMT is hypothesized to contribute to the development of interstitial fibrosis. Under certain conditions, TECs lose their epithelial phenotype and acquire a mesenchymal phenotype. To assess the prognostic relevance of EMT-related protein expression and investigate the pattern of EMT in renal allograft tissue, we analyzed protein expression and co-localization of tubular and mesenchymal markers in protocol renal transplant biopsies (Chapter 5). In chapter 6 the prognostic value of EMT-related markers was investigated at the mRNA level in protocol renal transplant biopsies.

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