Chapter 8

Summary and future perspectives
Summary

Kidney transplantation is inevitably faced with overcoming the immune response to the grafted organ, necessitating immunosuppressive therapy. Further, the immunogenicity of the graft is augmented by the initial ischemia-reperfusion injury, which negatively influences both acute and chronic renal graft failure. Because of its systemic nature, current immune suppression is a trade-off of efficacy against side-effects. Local, rather than generalized, immunosuppression or tolerogenic strategies may improve graft survival and quality of life of the transplanted patients. Targeted (over)expression of molecules that are able to modulate the immune reaction locally, in the graft, offers a strategy to shield the transplanted kidney against rejection, while leaving the immune system virtually unaffected. Gene therapeutic approaches may transform the theory of local immune suppression into reality. In addition, gene therapy may be instrumental in expanding the donor pool from non-heart beating donors and ameliorate ischemia-reperfusion injury in both living-related as well as deceased kidney transplantation. The aim of this study was to investigate (1) the potential of gene therapy with Interleukin-13, an immunomodulatory and cytoprotective cytokine, to mitigate renal I/R injury and acute kidney transplant rejection and (2) the potential of local immunosuppressive gene therapy in kidney transplantation.

The prerequisites of a successful gene therapy are an efficient, safe and selective vector, an appropriate technique and an appropriate gene (reviewed in chapter 2). Using both viral and non-viral vectors and employing various techniques, specific cell compartments of the kidney can be targeted. However, while plenty of studies show successful reporter gene delivery to the kidney, the number of functional studies with gene therapy in models of kidney disease in general and in transplantation in particular, is surprisingly low, presumably reflecting an insufficient transfection efficiency of the vectors used. From the available vectors for gene therapy, adenoviruses offer good perspectives, as they are able to infect a wide variety of cells, they have a relative high efficiency, are easy to produce on large scale and, important for kidney transplantation, they preserve their infectivity at low temperature. As a first step towards successful gene therapy for transplanted kidney, we employed in chapter 3 as vector a modified first generation adenovirus, which has an additional RGD sequence in the fiber knob. With this modification, both CAR (coxsackie adenovirus receptor)-dependent and CAR-independent (RGD-integrin-dependent) cell entry pathways are accessible for adenovirus, thus allowing enhanced infectivity. Indeed, the RGD-modification increased approximately 5-fold the transduction efficacy of the adenovirus, without enhancing the immune reactivity to viral genes in our model of rat kidney transplantation. Importantly, transgene expression was restricted to the renal graft, hence allowing selective, local gene therapy. With this vector and using an infusion-clamping technique, reporter gene (GFP) expression was found almost exclusively in the interstitial fibroblasts. As the interstitium of the renal graft is the stage of both immune infiltration, as well as long-term fibrosis, the most straightforward applications of fibroblast targeting in the context of kidney transplantation are local modulation of the immune microenvironment in the interstitium or blockade of fibroblast activation. As previously reported with the first generation adenoviruses (see chapter 2), the transgene expression was short-lasting (weeks), due to the adaptive immune response elicited by this type of adenovirus, making this vector suitable for short-term studies. We therefore aimed at investigating the potential of gene therapy in two of the most common acute events.
following kidney transplantation, which are the ischemia-reperfusion (I/R) injury and the acute rejection (AR).

Donor organs inevitably undergo ischemia followed by reperfusion, leading to variable degrees of damage (I/R injury). Especially long ischemia, as encountered in non-heart-beating donor kidneys, is accompanied by severe I/R damage, with both acute implications, i.e. delayed/non-graft functioning and acute rejection, and chronic consequences (chronic allograft nephropathy). To prevent or inhibit damage, one should know its cause and mechanism. For this purpose and taking advantage of the available microarray technique, we performed in chapter 4 an analysis of renal gene expression at several time points during a 3 week period after I/R, in a rat model. Our data confirmed changes in expression of genes already identified as being involved in I/R injury, such as transcription factors, stress response proteins, inflammation-related products and, at later time points, cell adhesion and extracellular matrix molecules. The two most important findings of this study are: (1) there is a quick and transient up-regulation of (mainly transcription) factors able to initiate not only an acute response to (hypoxic/oxidant) stress, but also a healing process through activation of inflammation and pro-fibrotic pathways; (2) factors (such as Egr-3 and Btg-2) that link innate and adaptive immunity come to expression after I/R injury, a process that was regarded till recently as being exclusively an innate immune response. In addition, in view of the following gene therapy studies, the pattern of several markers of renal I/R damage in time was defined: Kidney Injury Molecule (KIM)-1 for tubular damage; ED-1 (macrophages) for inflammation; α-smooth muscle actin (SMA) and collagen III for early and late fibrotic changes, respectively.

From a pathophysiologic perspective, a proper gene candidate to attenuate I/R injury and AR should combine anti-oxidant and anti-apoptotic properties with anti-inflammatory and immunomodulatory effects. Such a gene product is Interleukin(IL)-13. Previous data coming from studies in liver had documented strong protection from I/R with IL-13. As a proof of principle, chapter 5 investigates the effects of systemic (i.m.) IL-13 gene therapy in the renal I/R model described in chapter 4. Earlier studies showed that injection of adenoviruses in limb muscles (i.m.) is a feasible approach to deliver gene products systemically. Indeed, following i.m. injection of an RGD-adenovirus carrying the gene for IL-13, systemically circulating IL-13 levels were detectable for approximately 2 weeks. To ascertain that therapeutic levels of IL-13 were present at the time of I/R, i.m. delivery was performed 2 days prior to the I/R procedure. We demonstrated that IL-13 preserved renal histology and function after I/R. Specifically, IL-13 reduced tubulo-interstitial damage both on short and long-term (till day 14 after I/R), and limited neutrophil and macrophage infiltration by approximately 50% of their values in controls. This was associated with down-regulation of E-selectin and TNF-α mRNA levels, and, unexpectedly, with an almost unchanged expression of HO-1, which was described as a putative mediator of IL-13 protective effects in liver I/R. This apparent discrepancy remains to be clarified; a possible explanation is that tubular epithelial cells, which are the major renal source of HO-1 in this model, do not express IL-13 receptors. Thus, this study confirmed IL-13 as a novel and potent target for preventing renal I/R injury.

In a further step towards gene therapy for transplanted kidney, and taking advantage of the RGD-adenovirus vector described in chapter 3, we studied in chapter 6 the effects of local gene therapy with IL-13 on the acute rejection of the renal graft in rats. Systemic delivery of the IL-13 through i.m. RGD-adenovirus injection was also included in our study,
for comparison with regard to efficiency of the gene therapy approach. We showed that IL-13 reduced macrophage and CD8+ T cell infiltration, and down-regulated E-selectin, TNF-α and IFN-γ mRNA levels. Moreover, we found that expression of α-SMA was inhibited by approximately 40% compared to controls. Remarkably, the protection conferred by local as compared to systemic IL-13 therapy was similar, though elevated systemic IL-13 plasma levels were only found after i.m. gene therapy. One could speculate that the effects we found were exclusively locally-mediated. Or that the effects seen in the two treatments are (at least partially) the results of IL-13 acting on different (systemic vs. renal) cell types. However, the exact mechanisms involved herein remain to be investigated. We concluded that application of local IL-13 gene therapy appears as a feasible strategy to suppress the immune response locally, in the renal graft, and thereby to protect the graft against acute rejection.

Although the rat model is suitable for studying transplantation-related kidney damage, several other small species of mammals may provide new insights into better organ protection. Mammalian hibernation has been perceived as a natural model of repetitive I/R devoid of damage. As reduced endothelial-derived NO is incriminated as an important mediator of early injury during I/R injury10, whereas maintenance of eNOS activity trough, for instance, inhibition of Rho kinase11 attenuates I/R injury, we investigated in chapter 7 the expression of eNOS in hibernating ground squirrels. The main finding of this study was that eNOS expressed by endothelial cells of the peritubular capillaries and arterioles (“interstitial” eNOS) was unaffected by the hibernation, whereas glomerular eNOS was decreased throughout hibernation. In view of the fact that the tubules are the renal structures most sensitive to ischemic damage, and that eNOS-derived NO has vasodilatory and protective effects, it can be speculated that hibernating squirrels preserve their eNOS expression in the peritubular capillaries as a strategy to protect the tubules against I(/R) injury and to allow immediate restoration of tubular function during arousal. Is such a strategy applicable to the donor kidneys? Brodsky et al.12 showed that infusion of genetically modified cells (over)expressing eNOS into the I/R kidneys protected from I/R injury. Alternatively, NOS could be targeted to the endothelial cells through, for instance, an adenovirus coupled with an anti-(activated) endothelial cell marker, such as E-selectin.

Future perspectives

Improving the effects of IL-13 on AR through anti-T cell strategies

One of the most important finding of this thesis was that (over)expression of IL-13 in the donor kidney was able to diminish the acute immune response (chapter 6). Still, the effect on (CD8) T cell infiltration appeared less pronounced compared to that on macrophages. To improve these effects, one obvious approach is to combine IL-13 with a molecule that is able to inhibit chemotaxis or/and T cell proliferation or function. Such a molecule is 2,3-Indoleamine dioxygenase (IDO). IDO is the rate limiting enzyme in the catabolism of tryptophan, which is an amino acid essentially required for T cell survival, activation and proliferation13. Tryptophan starvation together with the tryptophan metabolites kynurenins may promote T cell apoptosis, induce T cell ignorance, anergy or generate regulatory T cells13,14. IDO is abundantly expressed in placenta (Figure A) and is crucially involved in maternal tolerance15. In addition, many tumors express IDO as a mechanism to escape the
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In our rat model of acute kidney transplant rejection, we found IDO expression to be limited to a few cells in the inflammatory infiltrates (Figure B). In view of this data, it is conceivable that overexpression of IDO in the renal graft using the available RGD-adenovirus vector system would protect the graft against rejection. In addition to its effects on T cells, IDO has also anti-oxidant properties and hence may further protect the graft from I/R injury, on top of the already documented effects of IL-13. A study regarding the effects of IDO gene therapy on AR of the transplanted kidney is ongoing.

**Gene therapy for chronic allograft nephropathy**

A major problem still to be solved in organ transplantation is chronic allograft failure. Following kidney transplantation, more than 50% of the patients develop progressive renal dysfunction, necessitating dialysis or a new graft within 10 years. As the pathogenesis of chronic allograft nephropathy (CAN) involves both immune and non-immune factors (see chapter 1), multiple therapeutic strategies have been attempted, none of them being yet effective. Can gene therapy take this challenge?

1. **long-term immune suppression or tolerogenic strategies**

A successful therapeutic strategy for CAN needs to interfere with its pathogenesis, to be local, long-term and non-toxic. As an alternative to the currently used systemic immune suppression, gene therapy offers the possibility of local immune surveillance. We (this thesis) and others (see chapter 2) have shown that local immune suppression through gene therapy can prevent AR. To combat chronic rejection, long-lasting vectors such as the helper-dependent (“gutless”) adenoviruses or adeno-associated viruses offer a good perspective. In the “gutless” adenovirus, the whole coding sequence of the viral genome has been removed, making the vector longer-lasting, safer (due to the lack of adaptive immune response against the viral genes) and increasing its coding capacity (so that several genes that target different pathways can be employed). As RGD-adenovirus is more effective in transducing the kidney than the unmodified virus, to optimize the “gutless” adenovirus, insertion of the RGD sequence into the fiber knob would be required. In addition, as repetitive immune injury is incriminated in the development of CAN, adjustable instead of continuous immunosuppression would likely be more appropriate. To adjust the level of immune suppression in chronic gene therapy, activation of interstitial fibroblasts may be used, as they act as “sensors” of local immune activation. Hereto, therapeutic genes, such as IL-13 and IDO, may be placed under the control of promoters with response elements of signaling routes that are activated in renal fibroblasts by the immune response, such as TGF-β or PDGF.

Another alternative to continuous systemic immune suppression is induction of tolerance (i.e. the lack of a destructive response against the graft in a fully immune competent recipient). Drug-free tolerance is rare in human kidney transplantation, as interruption of immunosuppression therapy usually leads to AR or chronic rejection. Besides donor bone marrow transplantation or infusion of immature donor dendritic cells prior to engraftment, gene therapy with blockers of co-stimulatory pathways of T cell activation, such as CTLA4Ig, have shown promising results. Notably, the tolerogenic effects of CTLA4Ig may be at least partially mediated by IDO.
2. anti-fibrotic strategies

Extensive tubulo-interstitial fibrosis is a hallmark of CAN. Incriminated herein are I/R damage, repetitive immune insults, proteinuria, hypertension. Irrespective of the trigger, the common pathway is the activation of interstitial fibroblasts (myofibroblasts)\textsuperscript{27,28}. With progressive fibrosis, tubular epithelial cells may also acquire a fibroblast-like phenotype (epithelial mesenchymal transition), further aggravating the fibrotic process\textsuperscript{29}. Blockade of fibroblast activation seems crucial to limit long-term fibrosis. We showed that (both local and systemic) IL-13 therapy inhibited early activation of fibroblasts, as documented by reduced β-SMA expression, though whether this was a direct effect of IL-13 on the interstitial fibroblasts or secondary to inhibition of inflammation needs further investigation. This was associated with reduced long-term fibrosis. With the RGD-adenovirus, most of the transduced cells were interstitial fibroblasts (see chapter 3). Thus, in addition to the IL-13 gene therapy, targeted blockade of, for instance, transforming growth factor (TGF)-β (through Smad7), or overexpression of anti-fibrotic factors, such as bone morphogenic protein (BMP)-7 or hepatocyte growth factor (HGF) via an RGD-“gutless” adenovirus seems feasible and may ameliorate long-term tubulo-interstitial fibrosis and thereby CAN.

Clinical perspectives

Kidney transplantation is nowadays generally viewed as the best therapeutic option for patients with end stage renal disease (ESRD). The life expectancy of patients with ESRD receiving a cadaveric kidney is twice as long as compared to patients remaining on chronic dialysis, even in elderly patients or after a long period of dialysis\textsuperscript{30}. The demand for donor kidneys grows along with the number of patients with ESRD\textsuperscript{31} and with ageing of the human population. Notwithstanding the increasing reliance on living donors, the waiting lists become longer every year. About 50% of the transplanted patients re-enter the waiting lists after 10 years due to CAN\textsuperscript{32}, adding to the already existing shortage of donors. Apart from efforts to increase the donor pool, extending the life span of the graft deserves full attention. This thesis shows that gene therapy can contribute to that in two ways: (1) optimize the outcome of cadaveric renal transplantation, by protecting the donor organ against I/R injury, and (2) improve the graft survival through local rather than systemic
immune suppression. With the development of more efficient, safer and longer-lasting vectors for gene therapy and with the advances in understanding the molecular pathways underlying (chronic) rejection, one expects that in short time we shall arrive at a point where clinically suitable vector(s) and gene products will be available. Of course, extensive testing in animal models is first needed before making the step to humans. Entering the clinical arena with gene therapy always imposes a hurdle, in view of the negative publicity on gene therapy brought by few cases of fatal side-effects induced by some of the vectors used\textsuperscript{33,34}. In addition, gene therapy with immunomodulatory agents may be considered with even more suspicion because of the recent TeGenero case\textsuperscript{35}, implicating that interference with mechanisms that control the immune system, such as regulatory T cells, may have dramatic side-effects. Finally, surprisingly, out of the about 1200 ongoing clinical gene therapy trials, none regards, to our knowledge, kidney transplantation. Consequently, while experimental gene therapy in kidney transplantation is advancing rapidly, its image and the lack of expertise in human trials may impede its entrance into the clinic. To prevent any delay, we better start preparing for clinical testing today. Not only for the benefit of the individual patient, but even more to cope with the ever increasing shortage of donors in an ageing population.
References


