The therapeutic potential of adenoviral gene therapy and angiotensine-(1-7) in proteinuric kidney disease
Wouden, Esther Anita van der

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Chapter 2

Approaches and methods in gene therapy for kidney disease

Els A. van der Wouden, Maria Sandovici, Robert H. Henning, Dick de Zeeuw, Leo E. Deelman

Abstract

Renal gene therapy may offer new strategies to treat diseases of native and transplanted kidneys. Several experimental techniques have been developed and employed using nonviral, viral and cellular vectors. The most efficient vector for \textit{in vivo} transfection appears to be adenovirus. Glomeruli, blood vessels, interstitial cells, and pyelum can be transfected with high efficiency. In addition, electroporation and microbubbles with ultrasound, both being enhanced naked plasmid techniques, offer good opportunities. Trapping of mesangial cells into the glomeruli as well as natural targeting of monocytes or macrophages to inflamed kidneys are elegant methods for site-specific delivery of genes. For gene therapy in kidney transplantation, haemagglutinating virus of Japan liposomes are efficient vectors for tubular transfection, whereas enhanced naked plasmid techniques are suitable for glomerular transfection. However, adenovirus offers the best opportunities in a renal transplantation setup because varying parameters of graft perfusion allows targeting of different cell types. In renal grafts, lymphocytes can be used for selective targeting to sites of inflammation. In conclusion, for both \textit{in vivo} and \textit{ex vivo} renal transfection, enhanced naked plasmids and adenovirus offer the best perspectives for effective clinical application. Moreover, the development of safer, nonimmunogenic vectors and the large-scale production could make clinical renal gene therapy a realistic possibility for the near future.
1. Introduction
Gene therapy holds the promise for treatment of renal diseases. Although, at first glance, gene therapy would be ideal for treating genetic deficiencies of the kidney, it could also be a suitable therapy for other renal diseases. Not only would this resolve issues such as systemic side effects of drugs needed to act only in the kidney\(^1\), it also may address the issue of therapy resistance to the currently available drugs, which is still a major problem in the field of nephrology\(^2\). Introducing therapeutic genes selectively into the kidney may overcome these problems and may have fewer side effects than conventional drugs. Gene therapy may also offer treatment opportunities with respect to kidney transplantation. Whereas acute rejection of the renal graft can mostly be prevented using immunosuppression, for chronic rejection, there is no effective treatment available and transfection of the graft during transplantation may provide new therapeutic possibilities.

To achieve therapeutic effects in the kidney, an efficient gene delivery to the site of action is needed. Moreover, to reduce side effects, the delivery should be selective, avoiding transfection of nontarget cells in kidney and other organs. Important factors determining the efficiency of the gene delivery are the vector that carries the gene and the route of administration. In addition, the gene chosen is crucial for therapeutic efficacy.

Several groups have published their data on new techniques in renal gene therapy using nonviral, viral and cellular vectors. Besides using different vectors, researchers have used both in vivo and ex vivo techniques. During in vivo transfection, the kidney is left in situ. In ex vivo transfection, a kidney is taken out from the body and, after transfection, transplanted.

This article compares efficacy, selectivity, and safety of the methods used in renal gene therapy including both in vivo and ex vivo techniques. For both techniques, nonviral, viral and cell vectors will be described and compared to conclude which techniques offer the best opportunities for clinical applications.

2. In vivo transfection
Besides the choice of the vector, the choice of the route of administration is a major determinant for effective and selective gene therapy. In addition, the application of different administration techniques allows targeting of different cell types in the kidney (see Fig. 1 for relative anatomic location). For in vivo transfection of the kidney, five routes of administration have been employed: renal arterial injection, renal venous injection, direct parenchymal injection, subcapsular injection, and retrograde delivery via the ureter (Fig. 1). In this section, administration of the different kinds of vectors through these techniques will be discussed and compared with respect to both efficacy and localisation.

2.1. Nonviral vectors
The simplest nonviral gene transfer system is the injection of naked plasmid DNA. Plasmids are independently replicating, circular, extrachromosomal DNA molecules naturally found in prokaryotes and eukaryotes. Transfection efficiency with plasmids is generally low and attempts have been made to
enhance the efficiency with several physical and chemical methods. Among the physical methods are electroporation, which creates temporary pores in the cell membrane, and the use of microbubbles together with ultrasound. Chemical approaches for enhancing plasmid transfection include liposomes, which are synthetic vesicles composed of a lipid bilayer. Encapsulation of DNA into liposomes allows uptake of DNA into the cell by endocytosis. In haemagglutinating virus of Japan (HVJ) liposomes, viral glycoproteins are incorporated in liposomes and used to enhance liposome-mediated gene transfer. The transfection efficiency and localisation of nonviral delivery techniques are summarised in Table 1 (see also Fig. 1).

![Figure 1](image)

**Figure 1.** Relative anatomic location of various cell types in the kidney. Schematic picture of a kidney and a microscopic image of the renal cortex.

### 2.1.1. Naked plasmids

Plasmids are able to transfect a broad range of cell types, they are easily produced in a large scale, the size of the gene insert may be large, and plasmids are very safe. However, efficiency is generally low and expression is only transient.

Direct injection of plasmid DNA into the renal cortex results in expression in the tubuli. However, although the plasmid was selectively applied in one kidney, expression was also found in the liver, heart, skeletal muscle, bladder, and, interestingly, uninjected contralateral kidney. Similar results were obtained for the kidney after systemic intravenous administration.

In 1999 Liu et al. published on the so-called hydrodynamic-based transfection technique. Rapid intravenous injection of a large volume of plasmids resulted in expression mainly in the liver, but expression was also found in the kidney. On rapid injection, a large volume is thought to accumulate in the inferior vena cava. Subsequently, the high hydrostatic pressure will force the DNA solution to flow into the tissues in a direction opposite to the normal circulation. Maruyama et al. adapted this technique for specific renal gene transfer. Rapid injection of a large volume of plasmids into the renal vein, with clamping of vein and artery, resulted in expression in cortical interstitial fibroblasts.
In summary, direct injection of naked plasmids into renal parenchyma will not result in efficient transfection of renal tissue. With injection of a large volume of plasmids into the renal vein, interstitial fibroblasts can be transfected.

Table 1. Success and localisation of in vivo renal gene transfer with nonviral vectors.

<table>
<thead>
<tr>
<th>Route of administration</th>
<th>Naked plasmids</th>
<th>Enhanced naked plasmids</th>
<th>Liposomes</th>
<th>HVJ-liposomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parenchyma</td>
<td>±</td>
<td>T⁴</td>
<td>-</td>
<td>⁸</td>
</tr>
<tr>
<td>Renal artery</td>
<td></td>
<td></td>
<td>± T¹⁰</td>
<td>† G¹¹</td>
</tr>
<tr>
<td>Renal vein</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ureter</td>
<td>+* IF⁶</td>
<td></td>
<td>± T¹⁰</td>
<td>† G¹¹</td>
</tr>
</tbody>
</table>

BV = blood vessels, G = glomerulus, IF = interstitial fibroblasts, T = tubulus, † = ß-actin promoter/CMV enhancer.

2.1.2. Enhanced naked plasmids

Because transfection with naked DNA is not efficient, several groups attempted to increase efficiency with physical methods. Electroporation creates temporary holes in the cell membrane, which allow plasmid DNA to enter the cell. After direct injection of plasmids in the renal cortex, electroporation of the kidney enhanced transfection to the tubuli⁷. However, transfection efficiency for the kidney was lower than for other urological organs as testis and bladder. Therefore, the kidney could not be transfected very efficiently with this technique. With injection in the renal artery and electroporation of the kidney, Tsujie et al. successfully transfected glomerular mesangial cells and, to a minor extend, tubular epithelial cells (TEC)⁹. In this study, 75% of the glomeruli were transfected. Retrograde injection of DNA into the ureter with electroporation delivered the DNA to the interstitial fibroblasts¹³. However, in this study, gene expression was not assessed since only the localisation of fluorescently labelled DNA was determined.

Another technique to enhance plasmid transfection is the use of microbubbles together with ultrasound. After application of ultrasound, microbubbles cavitate and release their DNA. Cavitation is also thought to cause a local shockwave, which results in the transient formation of holes in the cell membrane and improvement of cellular DNA uptake¹⁵. On arterial injection of plasmids in microbubbles and ultrasound exposure, almost all glomerular cells, vascular endothelial cells, interstitial fibroblasts, and, probably also the tubular cells were transfected. Using this technique, gene therapy with smad7, an endogenous inhibitor of the profibrotic transforming growth factor-ß (TGF-ß) signalling pathway, proved to reduce renal fibrosis in a rat model of ureter obstruction¹².

In summary, electroporation of the kidney, after injection of plasmids into the renal artery, is an efficient method for transfection of mesangial cells. In addition, the use of microbubbles and ultrasound forms an efficient technique for delivery of genes to glomerular cells, endothelial cells, and fibroblasts.
2.1.3. **Liposomes**

Liposomes are safe vectors for gene delivery because they are generally viewed as nontoxic and nonimmunogenic. They are easily produced and their inclusion volume allows different-sized genes up to the very large range. However, for *in vivo* gene delivery, liposomes are usually not as efficient as viral vectors and targeting of liposomes is difficult.

Boletta *et al.* developed a technique with renal arterial injection of liposomes, which resulted in weak gene expression in proximal tubular cells. Expression was generally absent in glomeruli and vasculature. Lai *et al.* also found expression in TEC on arterial injection and retrograde injection into the renal pelvis. With this technique, the gene for carbonic anhydrase, which is necessary for production of acid urine, was introduced in carbonic anhydrase knockout mice. After transfection, the mice gained the ability to produce acid urine. After direct injection of liposomes into the renal parenchyma, transfection was limited to the area surrounding the injection site.

In summary, transfection efficiency of TEC with liposomes is moderate to low. However, when genes encoding highly potent proteins are used, the low efficiency may still be sufficient to provoke therapeutic effects.

2.1.4. **Haemagglutinating virus of Japan liposomes**

The haemagglutinating virus of Japan (HVJ) is a parainfluenza virus expressing two glycoproteins on its surface that cooperate to achieve fusion of virus to the cell. In the HVJ liposome method, these glycoproteins are used to enhance liposome-mediated gene transfer. In addition, the high mobility group-1 is transfected together with the gene of interest. Cotransfection of high mobility group-1 enhances gene expression by several mechanisms, including facilitation of nuclear translocation of the DNA. HVJ liposomes are much more efficient vectors than normal liposomes, but the production is more complicated, expression is still only transient and HVJ liposomes are, in contrast to normal liposomes, immunogenic.

Injection of HVJ liposomes in the renal artery leads to gene expression in the glomerular mesangium and capillaries, whereas ureteral injection transfected interstitial fibroblasts. Expression of HVJ liposome transfection was successfully prolonged from 4 to 12 weeks by using an Epstein-Barr virus replicon vector.

In summary, HVJ liposomes are efficient vectors for transfection of the mesangium and interstitial fibroblasts after injection in the renal artery and the ureter, respectively.

2.1.5. **Conclusions**

In general, renal transfection with naked plasmids is not very effective. Electroporation and the use of microbubbles and ultrasound strongly enhance the transfection efficiency of naked plasmids and produce expression in mainly glomerular cells. The transfection success with liposomes is also generally low, whereas HVJ liposomes are more efficient vectors for renal gene therapy. With HVJ liposomes transfection is also mainly localised in the glomerulus. Therefore, for delivery to glomerular...
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cells electroporation, microbubbles, and HVJ liposomes are suitable delivery systems. For targeting tubular cells, nonviral vectors are not very useful. However, liposomes could be used but, due to the lower efficiency, probably only with highly potent genes. For targeting interstitial fibroblasts, injection of HVJ liposomes through the ureter or injection of a large volume of plasmids in the renal vein seems to offer the best perspectives.

2.2. Viral vectors

In general, viral vectors are much more efficient for gene therapy than nonviral vectors. For renal gene therapy, the most widely used viral vector is adenovirus. Adenoviral vectors are able to transfect a wide range of cells, both dividing and nondividing cells, with high efficiency. However, adenoviral transfection is only transient. Other viruses used for renal gene therapy are adeno-associated viruses (AAV), retroviruses and lentiviruses, since these viruses provide long lasting expression, in contrast to adenovirus. The transfection efficiency and localisation of viral delivery techniques are summarised in Table 2 (see also Fig. 1).

<table>
<thead>
<tr>
<th>Route of administration</th>
<th>Adenovirus</th>
<th>Adeno-associated virus</th>
<th>Retrovirus</th>
<th>Lentivirus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parenchyma</td>
<td>-</td>
<td>±</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td>Renal artery</td>
<td>±</td>
<td>T^{24}</td>
<td>+^</td>
<td>T^{25}</td>
</tr>
<tr>
<td></td>
<td>+^</td>
<td>BV^{26}</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td></td>
<td>±</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+^</td>
<td>I^{27}</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>++</td>
<td>G^{28}</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>++^§</td>
<td>G^{29}</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>++^§</td>
<td>G^{30}</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>++^§</td>
<td>G^{31}</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>++^§</td>
<td>BV^{32}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Renal vein</td>
<td>+</td>
<td>P, T^{24,27}</td>
<td>-</td>
<td>CD^{23}</td>
</tr>
<tr>
<td>Ureter</td>
<td>+</td>
<td></td>
<td>±</td>
<td>T^{23}</td>
</tr>
</tbody>
</table>

BV = blood vessels, CD = collecting ducts, G = glomerulus, I = interstitial cells, P = pyelum, T = tubulus, ^ = β-actin promoter/CMV enhancer, @ = polystyrene microspheres, * = Ad-RGD, $ = clamping of the renal vein, § = slow infusion, ∞ = perfusion.

2.2.1. Adenoviruses

Adenovirus is widely used for in vivo gene transfer because it can transfect numerous cell types, both dividing and nondividing cells. In addition, the virus can be easily grown in high titers. However, adenovirus has several drawbacks such as a transient transfection, a natural tropism for the liver, and
an immune response to the viral proteins and the viral particles. In a newer generation of adenovirus, the 'gutless' adenovirus, the entire coding sequence of the adenoviral genome has been deleted. This adenovirus has been shown to be far less immunogenic and therefore results in a longer duration of expression\textsuperscript{33}. Much progress has also been made in retargeting adenovirus to other receptors besides coxsackievirus and adenovirus receptor (CAR), its natural receptor, by modification of the coat proteins and complexation with other molecules, such as bispecific antibodies\textsuperscript{34}.

The first article on renal adenoviral gene therapy was published by Moullier et al.\textsuperscript{24}. Injection of the virus into the renal artery by these and other researchers\textsuperscript{35} resulted in weak expression in proximal tubuli. Zhu et al. developed a more optimised technique, with prolonged incubation of the virus and cooling of the kidney to reduce ischemic damage\textsuperscript{26}. In contrast to Moullier's data, expression was found mainly in the vascular endothelial cells. In a canine model, with prolonged exposure by clamping the renal vein, Chetboul et al.\textsuperscript{27} transected interstitial and endothelial cells using an arterial injection. However, in dogs and pigs, a single injection into the renal artery did not yield any transfection of the kidney\textsuperscript{27,28} possibly due to a relatively low dose. Perfusion of the porcine kidney\textsuperscript{28,30} or slow infusion in rat and rabbit kidney\textsuperscript{29,30} enhanced transfection efficiency but also changed the localisation of expression to the glomerulus. However, at a lower dose, transfection of rabbit kidney was located in the arterial endothelium\textsuperscript{30}. The percutaneous catheterisation technique for infusion of adenovirus in the renal artery of rabbits developed by this group is more adapted to a clinical application\textsuperscript{30}.

With retrograde injection of adenovirus in the ureter, Moullier et al. found a strong expression in the papilla and in the tubular cells in the medulla\textsuperscript{24}. In a canine model, expression was located in the pyelum and distal tubules\textsuperscript{27}. With the gene for aquaporin-1, which is important for proximal tubular water transport and the concentration of urine, transfection was found in papilla and collecting ducts of aquaporin-1 knockout mice\textsuperscript{19}. Terada et al. successfully enhanced transfection after retrograde administration of adenovirus by using electroporation and ligation of the ureter to prolong exposure\textsuperscript{36}.

The simplest way of delivering adenovirus to the kidney is probably direct injection into the renal parenchyma. However, homogenous expression has not been achieved by this technique. Although direct injection of adenovirus into the interstitium yielded some renal transfection, expression was limited to the injection site\textsuperscript{19,20}.

A very simple strategy to target adenovirus to the glomerulus was published by Nahman et al. Adenovirus was complexed to polystyrene microspheres and injected into the renal artery. The microspheres were trapped in the glomeruli and in this way endothelial and mesangial cells could be transected\textsuperscript{31}. Moreover, the kidneys were devoid of any histological damage or signs of ischemia.

A very promising strategy to enhance transfection of the kidney is the use of modified viruses. The adenoviral vectors, used for gene therapy, bind to their primary receptor, the CAR receptor. After binding, internalisation occurs by binding of the penton base of the virus to the integrin receptor. However, the CAR receptor may be scarce in several tissues, including the kidney\textsuperscript{32}. McDonald et al. used an Arg-Gly-Asp (RGD)-modified adenovirus that, in addition to CAR receptors, also binds to $\alpha_v\beta_3$-integrins, which are more abundant in renal tissue\textsuperscript{38}. After renal arterial injection of this RGD
Adenovirus, expression was, as with normal adenovirus, found in the vascular endothelium. However, the viral dose could be lowered eightfold and expression was more located in the cortical region.

In summary, adenovirus is an effective vector for renal gene therapy, but expression needs to be enhanced by prolonged incubation or perfusion of the kidney. Rapid arterial injection induces variable expression in the tubular epithelium, vascular endothelium, or interstitium. In contrast, perfusion and slow infusion of adenovirus in the renal artery result in glomerular expression. Papilla and tubular cells can be targeted with a retrograde injection of adenovirus.

2.2.2. Adeno-associated viruses
Adeno-associated virus (AAV) type 2, a nonpathogenic human parvovirus, is nowadays the most often used paroviral vector for gene therapy. AAV is able to infect both dividing and nondividing cells and provides long-term expression by integration into the host genome. Besides, recombinant AAV does not contain any viral genes and therefore does not generate an immune response. The major disadvantages of AAV are formed by the complex production of virus and the limited size of the inserted gene.

Studies on renal in vivo transfection with AAV are limited but consistent. Injection of the virus in the renal artery with clamping for 5 or 45 minutes resulted in expression in the proximal tubule cells. In addition, after direct injection in the renal parenchyma, expression was located in the proximal tubule. Using either technique, expression was absent in glomeruli, blood vessels, and interstitial cells. However, with direct parenchymal injection, the transfection was limited to the injection site.

Interestingly, in this study, the expression lasted for at least 3 months. Therefore, proximal tubule cells can be lastingly transfected by an injection of AAV in the renal artery.

2.2.3. Retroviruses
Retroviruses can randomly integrate into the host genome and result in stable, long-term expression. However, for stable integration, cell division is necessary. Therefore, retroviruses are unable to transfect nondividing cells. Immune responses are largely absent, but generation of viral stocks with high titer is difficult. The Moloney murine leukemia virus (MMLV) is most widely used for gene therapy studies, however the use is declining over the years.

In the rat kidney, MMLV-mediated gene transfer was achieved after induction of cell division by administration of a nephrotoxic dose of folic acid. MMLV was directly injected into the kidney, which resulted in expression in only few TECs in approximately 50% of the transfected kidneys.

Therefore, retroviruses do not seem to be very suitable vectors for renal gene therapy because adult kidney cells have a low mitotic index and cell division needs to be artificially increased. However, in pathologic conditions of increased cell division, retroviruses may prove to be suitable vectors.
2.2.4. Lentiviruses

Among the most frequently used lentiviruses, a class of retroviruses, are the human immunodeficiency virus (HIV) type 1 and simian immunodeficiency viruses. Lentiviruses are able to transfect nondividing and dividing cells and provide long-term expression by stable integration into the host genome. Although lentiviruses have become safer and easier to produce by recent development of replication incompetent vectors and stable packaging cell lines, potential reversal to the pathogenic wild-type virus is a major drawback\(^3\).

Renal gene transfer with lentivirus has been extensively studied by Gusella et al. Expression was achieved after injection into the renal artery or vein, retrograde infusion into the ureter and after parenchymal injection. However, renal arterial and venous injection yielded patchy transfection of only a few cells in the collecting ducts, whereas retrograde administration induced weak but more diffuse transfection of proximal tubules. Expression in proximal tubule cells after parenchymal injection was stronger but limited to the area near the injection site. Expression lasted for at least 3 months. Further optimisation of transfection efficiency proved to be difficult because of the inability to produce high titer stocks of lentivirus\(^2\).

In summary, due to a low efficiency in transfecting kidney cells, lentiviruses do not seem to be promising vectors for renal gene therapy.

2.2.5. Conclusions

Although renal gene therapy with retroviruses, including lentiviruses, provides long-lasting expression, efficiency is too low. Adenoviral transfection is the most extensively studied. Administration through the renal artery seems to be the most efficient strategy. However, prolonged exposure by clamping the renal vein or by perfusion or slow infusion is necessary to achieve a higher expression. When clamping of the renal vein is employed, transfection is mainly found in blood vessels and interstitial cells, whereas slow infusion or perfusion with adenovirus results in glomerular expression. Retrograde injection of adenovirus through the ureter results in an efficient transfection of the pyelum. To achieve tubular transfection, adenovirus is not the most optimal vector, but AAV seems to offer an effective alternative.

2.3. Genetically modified cells

In this approach, vector cells are transfected in vitro and then transferred into the animal. Depending on the targeted structure within the kidney, mesangial cells, monocytes and macrophages, and TECs have been studied. The transfection success and localisation of genetically modified cells are summarised in Table 3 (see also Fig. 1).

2.3.1. Mesangial cells

Genetically modified mesangial cells are attractive vectors to selectively express genes within the glomerulus. Extensive work using a mesangial cell vector system has been carried out by Kitamura et al. In normal rats, expression was observed for 4 weeks after injection of the cells into the renal artery\(^3\).
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Approximately 60% of the glomeruli showed expression. Moreover, *in situ* amplification and longer expression could be achieved by preconditioning of the glomeruli. When the cells were transferred into damaged glomeruli, *in situ* expression increased 7-12-fold through mesangial cell proliferation and lasted for up to 8 weeks. Therefore, this approach seems to be suitable especially for treating glomerulonephritis.

To overcome rejection of the vector, the use of autologous mesangial cells cultured from renal biopsy specimens has been proven feasible. However, this approach is laborious, since it requires isolation, growth and transfection of mesangial cells from each individual.

Table 3. Success and localisation of in vivo renal gene transfer with genetically modified cells

<table>
<thead>
<tr>
<th>Route of administration</th>
<th>Mesangial cells</th>
<th>Monocytes/macrophages</th>
<th>TEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal artery</td>
<td>+ G&lt;sup&gt;39,40&lt;/sup&gt;</td>
<td>-&lt;sup&gt;*&lt;/sup&gt; G&lt;sup&gt;41&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Intravenous</td>
<td>+ G&lt;sup&gt;42&lt;/sup&gt;</td>
<td>I&lt;sup&gt;43&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Subcapsular</td>
<td></td>
<td>± I&lt;sup&gt;44&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

G = glomerulus, I = interstitial cells, * = β-actin promoter/CMV enhancer.

2.3.2. **Monocytes and macrophages**

Gene-engineered monocytes and macrophages have been used as a site-specific gene delivery system into inflamed kidneys because of their natural migration following inflammatory chemotactic signals. The use of autologous cells attenuates the risk of vector rejection. Gene transfer into monocytes or macrophages can be achieved via several methods including the use of polylysinated mannose, retroviruses, and adenoviruses. Of these, the viral approach seems to be the most efficient one. Adenoviral transfected macrophages are less immunogenic than adenovirus alone. However, viral transfection activates macrophages in a dose-dependent manner, and this could induce additional renal injury.

Since monocytes and macrophages follow inflammatory chemotactic signals, their localisation after injection depends on the disease model chosen. Using injections of lipopolysaccharide, which stimulates the glomerular expression of the chemotactic ICAM-1, Yokoo *et al.* successfully targeted intravenously administered bone marrow derived cells, naturally expressing ICAM-1 ligands, to the glomerulus. In a unilateral ureteral obstruction model, characterised by interstitial inflammation, intravenously administered bone marrow cells migrated selectively into the inflamed interstitium. After injection of activated genetically modified macrophages into the renal artery of rats with acute glomerular inflammation, over 80% of the glomeruli contained transfected macrophages.

In summary, because of their natural migration to inflammatory sites, monocytes and macrophages are suitable vectors for targeting inflamed kidneys. However, the laborious procedure of culturing and transfecting cells from each individual may hamper clinical applications of this technique.
2.3.3. Epithelial cells
TECs, stably transfected \textit{ex vivo} using a replication-deficient retrovirus, have also been used as a gene delivery system. The implantation of TECs carrying proinflammatory cytokine genes under the renal capsule led to increased circulating levels of cytokines for at least 4 weeks\textsuperscript{44}. The effect of cytokine secretion was limited to the transfected kidney, probably because high levels of cytokines are required locally to induce a functional effect. However, the cell infiltration as a result of cytokine secretion was not uniformly distributed within the kidney, being most prominent in the area surrounding the cell implantation.

2.3.4. Conclusions
Glomerular targeting can be easily obtained by renal arterial injection of \textit{in vitro} transfected mesangial cells. For targeting to inflamed kidneys the natural migration of monocytes or macrophages to inflammatory sites provides good opportunities. However, culturing and transfection of autologous cells is a laborious procedure, which may complicate clinical application.

3. \textit{Ex vivo} transfection
The transplanted kidney is a particularly appropriate target for gene therapy. Gene delivery can be performed \textit{ex vivo}, allowing manipulation of the transfection conditions and precluding transfection of other organs. A multitude of genes could be employed to influence both immune and nonimmune factors involved in transplant related pathology, thereby preventing graft failure. Both nonviral and viral vectors have been used to transfer genes into the transplanted kidney.

3.1. Nonviral vectors
For gene therapy in models of renal transplantation, several nonviral vectors have been investigated. Naked plasmids have been injected in the renal artery but showed to be ineffective as gene delivery system into the transplanted kidney\textsuperscript{45}. From the enhanced naked plasmid techniques, both electroporation and the use of microbubbles together with ultrasound have been applied. Liposomes and HVJ liposomes have been examined in transplanted kidneys. The efficiency and localisation of the nonviral delivery systems used in kidney transplantation are summarised in Table 4 (see also Fig. 1).

3.1.1. Enhanced naked plasmids
Two methods have been employed to enhance naked plasmid transfection efficiency in kidney transplantation: electroporation and the use of microbubbles with ultrasound. Isaka \textit{et al.} applied electroporation to facilitate hepatocyte growth factor (HGF) gene delivery in a porcine model of kidney transplantation\textsuperscript{46}. \textit{Ex vivo}, the renal vein was clamped and naked plasmids were infused into the renal artery followed by electroporation. HGF mRNA production was still present 6 months after transfection, confined to the transplanted kidney. Interstitial fibrosis, which is one of the major histological features of
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Table 4. Success and localisation of ex vivo renal gene transfer with nonviral vectors

<table>
<thead>
<tr>
<th>Route of administration</th>
<th>Naked plasmids</th>
<th>Enhanced naked plasmids</th>
<th>Liposomes</th>
<th>HVJ-liposomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal artery</td>
<td>-</td>
<td>++?45</td>
<td>-</td>
<td>++?48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>?47</td>
<td></td>
<td>T49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G, T46</td>
<td>+?</td>
<td>BV50</td>
</tr>
<tr>
<td>Intravenous</td>
<td>++?51</td>
<td></td>
<td>+?</td>
<td></td>
</tr>
</tbody>
</table>

BV = blood vessels, G = glomerulus, T = tubulus, $ = clamping of the renal vein.

chronic renal graft failure, was reduced through HGF gene therapy. When in vivo infusion of the fluorescently labelled DNA together with an echo-contrast agent containing microbubbles was combined with ex vivo exposure of the kidney to ultrasound, fluorescence was found in more than 70-80% of the glomeruli and most tubular cells. With this technique, transfection of nuclear factor κB (NFκB), a transcription factor involved in the onset of acute rejection, improved the function and the histology of the graft and prolonged survival in a rat renal transplantation model47.

Although localisation of the gene expression is not very well studied, both electroporation and the use of microbubbles with ultrasound proved to be suitable vector systems for the transplanted kidney because relevant therapeutic effects have been shown with both techniques.

3.1.2. Liposomes

The research of Benigni et al. in a rat kidney transplantation model showed that cationic polymer polyethylenimine liposomes are toxic for kidneys. Lowering the dosage did improve toxicity, but transgene expression was not detected in the graft48.

However, different cationic liposome systems showed to be effective in reducing ischemia-reperfusion injury of the renal graft. In situ perfusion of the donor kidney with liposomes containing fluorescently labelled NFκB decoy oligodeoxynucleotides (ODN) at 37 °C yielded a fluorescent signal in most of the peritubular capillaries for 1-3 days50. Early inhibition of the NFκB activation decreased adhesion molecule expression and monocyte infiltration within the first 3 days after transplantation. Liposome-delivered antisense ODN for ICAM-1, when injected intravenously 6 h before transplantation, improved immediate graft function and histology in a model of kidney autotransplantation51. ICAM-1 ODN presumably prevented leukocyte adhesion to the endothelium, which plays an essential role in ischemia-reperfusion injury of the graft.

Although the localisation of the expression with liposome-mediated gene delivery systems is poorly investigated, gene therapy with liposomes shows short-term effects in kidney transplantation. Due to the brief duration of expression, liposomes are no suitable vectors for long-term treatment.

3.1.3. Haemagglutinating virus of Japan liposomes

The HVJ liposomes seem to provide an efficient system for transfection of the kidney in a cold environment. When slowly injected into the renal artery of the donor rat followed by incubation at 4 °C,
Chapter 2

the HVJ liposomes delivered the gene into the TECs\textsuperscript{49}. Moreover, the transfer of anti-apoptotic gene Bcl-2 by this system allowed prolongation of preservation time and improved cell viability in the graft, thereby preventing primary nonfunction after transplantation.

3.2. Viral vectors

In renal transplantation, adenovirus is the most widely used viral vector for gene therapy. In a special approach of transplantation, a retrovirus vector system has also been employed to genetically modify embryonic metanephric tissue, which was then transplanted into the kidney of neonatal mice. The reporter gene expression was mostly found in glomerular epithelial cells of the embryonic tissue\textsuperscript{52}. The transfection success and localisation of the viral delivery systems used in kidney transplantation are summarised in Table 5 (see also Fig. 1).

<table>
<thead>
<tr>
<th>Route of administration</th>
<th>Adenovirus</th>
<th>Retrovirus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal artery</td>
<td>+∞ T\textsuperscript{53}</td>
<td>- 54</td>
</tr>
<tr>
<td></td>
<td>++∞ G\textsuperscript{28:54}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>++∞ BV, T\textsuperscript{55}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+∞ T\textsuperscript{48}</td>
<td></td>
</tr>
<tr>
<td>Subcapsular</td>
<td>+ G\textsuperscript{52}</td>
<td></td>
</tr>
</tbody>
</table>

BV = blood vessels, G = glomerulus, T = tubulus, & = hybrid Adenovirus-polylysine, ∞ = perfusion.

3.2.1. Adenoviruses

Adenovirus is the most used vector for gene transfer to the renal graft. For transplantation, adenovirus has the distinct advantage that it can transfect several cell types at low temperature\textsuperscript{56}. This allows pretransplantation gene transfer to be carried out during the process of cold preservaton. The transgene delivered through an adenoviral vector can express its product for 1-3 weeks\textsuperscript{24,28,29}. In protocols aiming at influencing acute rejection, which in small animals develops within the first 1-2 weeks after transplantation, delivery via adenovirus should provide sufficient time for transgene product effects. However, for chronic graft failure, the ‘gutless’ adenovirus would be a more appropriate choice, due to its longer expression of months\textsuperscript{57,58}. In allotransplantation, the initial immunosuppressive treatment can also prolong the adenoviral-delivered gene expression through inhibition of the immune response against the viral proteins\textsuperscript{59}.

In 1996, Zeigler et al. reported for the first time successful gene transfer into isolated human kidneys using a hybrid adenovirus-polylysine-DNA complex as vector, which was delivered by pulsatile perfusion for 2 h at 4 °C. The reporter gene localised mainly in the proximal tubules\textsuperscript{53}. In pigs, high rate perfusion of the explanted kidney at room temperature, even for 17 h, did not yield any gene expression
at the end of the perfusion period\textsuperscript{54} probably because a higher temperature is required for the kidney cells to efficiently express viral proteins. Indeed, increasing the perfusion temperature to 37 °C resulted in marked expression of the gene in approximately 80% of glomeruli after 12 h of perfusion\textsuperscript{28,54}. In dog kidneys perfused for 24 h at 32 °C, gene expression localised to blood vessels and tubuli, depending on the virus dose\textsuperscript{55}. Proximal and distal tubule expression was also achieved in rats, whose kidneys were perfused for 1 h at 4 °C\textsuperscript{48}. However, gene expression was also found in the contralateral kidney, liver, and lung probably because the kidney was not adequately flushed before transplantation.

Adenoviral-mediated gene transfer proved its efficacy in prolonging rat renal allograft survival when anti-inflammatory molecules such as IL-10, IL-12p40, TNFRp55-Ig\textsuperscript{60}, IL-4\textsuperscript{61} and CTLA-4Ig, which is a blocker of T cell activation\textsuperscript{45}, have been delivered into the transplanted kidney. However, long-term graft function was not improved probably due to reduction of the therapeutic gene expression in time\textsuperscript{60}. Therefore, a long lasting, nonimmunogenic vector would be more suitable for gene transfer when one aims at preventing chronic graft rejection.

Summarising, adenovirus is the most efficient viral vector in kidney transplantation. Depending on different parameters of graft perfusion, such as temperature, perfusion pressure, and viral dose, targeting to glomeruli, blood vessels, or tubuli can be achieved. The ‘gutless’ adenovirus holds the promise for long lasting gene expression required for preventing chronic graft failure through gene therapy.

### 3.3. Genetically modified cells

Autologous bone marrow cells, dendritic cells, and T-cells are the most important cell vectors used for transfection of the renal graft. The success and localisation of these cell delivery systems are summarised in Table 6 (see also Fig. 1).

Autologous cells are appealing vectors for kidney transplantation because the risk of vector rejection is reduced. Transplantation of autologous bone marrow, retrovirally transfected with allogeneic donor-type major histocompatibility complex II genes, induced prolonged renal graft survival in pigs\textsuperscript{62}.

Dendritic cells are classically regarded as antigen-presenting cells required for initiation of the primary T-cell response but are also thought to be important for induction of immunological tolerance\textsuperscript{63}. Enhancement of their potential to induce tolerance can be achieved by genetically modifying dendritic cells to express immunomodulatory molecules such as IL-10, TGF-β or CTLA-4Ig. In mice, increased allograft survival was achieved using intravenously administered donor dendritic cells transfected to express IL-10 and TGF-β\textsuperscript{64}.

The use of T-lymphocytes as vector is a novel strategy for antigen-specific targeting in kidney transplantation, aiming at inducing graft tolerance in the recipient. Priming of the T-cells \textit{in vitro} with alloantigens leads to generation of T-cell lines with a defined antigen specificity, which may subsequently be transfected using a retrovirus. When administered intravenously, alloantigen-specific, genetically-engineered T-cells migrate selectively into the allograft, where the alloantigen is expressed, especially the tubular region but also the glomeruli being infiltrated\textsuperscript{65}. Furthermore, alloantigen-specific
activation increases transgene expression *in vivo*\(^6\). However, involvement of the transferred T-cells in the rejection process might be a major drawback of this vector system.

In summary, cell vectors have the advantage of selective targeting to the site of the immune reaction, which make these vectors particularly suitable vectors for transplanted kidneys. However, the labour-intensive techniques and the possible involvement of the vectors in the rejection process are the main disadvantages of cell vectors.

Table 6. Success and localisation of ex vivo renal gene transfer with genetically modified cells

<table>
<thead>
<tr>
<th>Route of administration</th>
<th>Bone-marrow cells</th>
<th>Dendritic cells</th>
<th>T-cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow Tx</td>
<td>+</td>
<td>?(^62)</td>
<td></td>
</tr>
<tr>
<td>Intravenous</td>
<td>+</td>
<td>+ (?(^64))</td>
<td>G, T(^65)</td>
</tr>
</tbody>
</table>

G = glomerulus, T = tubulus.

3.4. Conclusions

Naked plasmids are ineffective as vector for *ex vivo* transfection. Liposomes provide only short-term gene expression in the kidney graft and, although generally thought of as being nontoxic, some of them are toxic. Transfection is improved through enhanced naked plasmids and HVJ liposomes, which are most suitable for targeting the tubuli. By far, the most efficient and the most widely used vector is adenovirus. Varying the parameters of graft perfusion, such as temperature, perfusion pressure, or viral dose, allows both targeting to different cell types within the kidney and improving the transfection efficiency. The ‘gutless’ adenovirus holds the promise for long lasting gene expression required for preventing chronic graft failure through gene therapy. Cell vectors, such as bone marrow cells, dendritic cells, and T-cells, have the advantage of selective targeting to the site of the immune reaction. However, these techniques are labour intensive and therefore possibly difficult to apply in a clinical setting. Moreover, tight regulation of the immune function of the cell vectors would be necessary to preclude their involvement in the rejection process.

4. Limitations in comparing transfection efficiencies

Evaluation of a new gene therapy technique usually takes place by using a reporter gene. However, different reporter genes are employed. The bacterial β-galactosidase gene is the most often used one. However, the kidney has some endogenous β-galactosidase activity. Although it is possible to inactivate mammalian β-galactosidase selectively by heating\(^67\) or incubation at weakly alkaline pH\(^68\) or to use nuclear targeting of β-galactosidase\(^69\), it may not be the optimal reporter gene for evaluating renal gene transfer.

The gene encoding green fluorescent protein (GFP) is an optimal reporter gene neither. Since the kidney displays a high autofluorescence, background fluorescence is high and transfection efficiency
difficult to measure. Staining with anti-GFP antibodies could overcome this problem. However, determination with antibodies represents only an indirect evaluation of expression.

For quantification of transfection efficiency luciferase would be the most optimal reporter gene. However, for localisation an indirect detection with antibodies is needed.

Due to differences in background signal, different reporter genes may result in different transfection efficiencies. Therefore, a comparison of techniques is difficult when different reporter genes are used.

Another important factor is the use of different promoters. For transfection experiments, usually the cytomegalovirus (CMV) promoter, which yields high expression in almost all cells, is used. However, there are indications that this is not the most optimal promoter in the kidney. In a direct comparison Maruyama et al. used a β-actin promoter in combination with a CMV enhancer with much more success than the CMV promoter. In general, renal transfection with naked plasmids in ineffective unless the β-actin promoter is used (Table 1). Also the success of the technique with electroporation could be based on the use of this promoter. The same may be true for the HVJ liposome technique.

Therefore, when evaluating articles on renal gene therapy, one should consider the reporter gene and the promoter used because these factors may also influence the expression of the transgene.

5. Conclusion

Although clinical renal gene therapy is not yet a reality, several techniques reviewed in this article show to be promising for future therapeutic applications both for in vivo and for ex vivo gene therapy (e.g. in the context of transplantation). Enhanced naked plasmids and adenovirus are the most effective vectors and have potential as vectors for clinical gene therapy because delivery through intra-arterial catheters and large-scale production of plasmids and adenovirus are feasible. However, safety issues remain a drawback for the clinical use of the currently available adenoviral vectors. In addition, the natural tropism of adenovirus for hepatocytes complicates selective renal expression. In this perspective, enhanced naked plasmid techniques have the benefit of combining good efficacy with relatively few safety issues. In addition, the development of less immunogenic adenoviruses may bridge the gap between experimental and clinical application.

As far as cells as vector are concerned, genetically modified immune cells are quite interesting because they have sites of inflammation as their natural target. They are obviously less useful for renal diseases that have no major inflammatory component.

For the near future, enhanced naked plasmid techniques and the application of less immunogenic adenoviruses appear to remain the gene therapy modus of choice for (new) clinical applications.
References


Approaches and methods in gene therapy


