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PERSPECTIVES IN BASIC SCIENCE

Strategies of gene transfer to the kidney

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Strategies of gene transfer to the kidney. Kidney targeted gene transfer has been a realistic goal for many researchers since 1991, but unfortunately, to date there is no reliable gene transfer technique for gene therapy of renal diseases. However, at the experimental level, several *in vivo* gene transfer methods have attempted to target certain renal structures, for example, the HVJ-liposome method and renal perfusion of adenovirus for glomerular cells, intravenous injection of oligonucleotides (ODNs) for proximal tubule, intra-arterial injection of adenovirus followed by cold incubation with a vasodilator for interstitial vasculature of the outer medulla, and adenoviral injection into the renal pelvis for the inner medullary collecting duct. As an *ex vivo* gene transfer method targeting the glomerulus, the transfusion of genetically-modified mesangial cells has been attempted. Implantation of genetically-modified tubular epithelial cells into the subcapsular region has been employed for *ex vivo* transfection to the interstitium. Gene therapy has focused particularly on the transplanted kidney, where an exogenous gene can transferred in advance. In the future, an inducible system and individual cell targeting strategy should be developed. The improvement of gene transfer techniques, especially vectors for delivering genes, is crucial. The potential application of gene transfer technologies is enormous while the therapeutic approaches have just begun to be explored. Therapeutic interventions of the process of progression of glomerulonephritis in the rat have been directed towards inhibiting the actions of growth factors. Obviously, molecular biological intervention is coming of age and there is a tremendous excitement over its potential. We believe that gene transfer techniques will become common tools for the dissection of molecular aspects of diseases and possibly for gene therapy in the field of nephrology.

The first gene therapy in human beings was done in a girl with adenosine deaminase (ADA) deficiency in 1990 [1]. This therapy succeeded in the long-term expression of the transferred ADA gene in T lymphocytes, and the girl who suffered from severe immunodeficiency was allowed to return to school. This happy story was a tantalizing step forward in the application of basic research to clinical problems, and since then general interest concerning gene therapy has exploded. From the first clinical trial seven years ago, more than 200 protocols for human gene therapy have been approved and 2100 patients were recruited for gene therapy by the end of 1996 [2]. Gene therapy was originally a clinical strategy in which genetic material is transferred to somatic cells to correct an inherited genetic disorder. The rationale of somatic gene therapy is the correction of the cause(s) of diseases at the most fundamental level. The application of gene therapy has been extended to the treatment of acquired diseases including cancer, human immunodefficiency virus (HIV) infection and vascular diseases. To date, two thirds of the diseases addressed by gene therapy have been malignancies while only 10% have been congenital deficiencies [2].

Potentially, gene therapy may provide: (*1*) a correction of cellular dysfunction by expressing the deficient gene, (*2*) the addition of a new function for a cell by transferring an exogenous gene and (*3*) inhibition of unfavorable action of a cell by introducing a counteracting gene. The most attractive aspect of gene therapy is the introduction of a functional molecule into a cell, because transferring a protein across the plasma membrane of a targeted cell is rather difficult compared to transferring DNA. While clinical trials examining human gene therapy are apparently increasing at a remarkable rate, significant information regarding the long-term efficacy and adverse consequences are still generally lacking. One cutting report on gene therapy was released by Orkin and Motulsk from the National Institutes of Health (Bethesda, MD, USA) in 1995 [3]. They pointed out important issues on gene therapy, and in particular, the weakness of the basic research on gene therapy. They believed that more attention should be paid to comprehension of disease pathophysiology, to extend the knowledge of which cells should be targeted and which gene(s) should be corrected for the physician to accomplish the most effective therapy. The development of gene transfer techniques, especially vector(s) for delivering a gene, is crucial for the improvement of gene transfer efficacy. As nephrologists, we should accept these criticisms about gene therapy calmly and make every effort to promote fundamental research. Although the technologies for gene therapy are not fully developed, the logic of gene therapy continues to be compelling. Recent reviews have summarized the progress of human gene therapy [4–9], and others have focused on its application to renal diseases [10–14].

In contrast to somatic cell gene manipulation, germ line gene manipulation is ethically prohibited for human gene therapy. Germ line mutation has been used for producing new phenotypes of animals. The mutation can be established by nonhomologous or homologous recombinations. The former is used as a strategy to produce transgenic mice and the latter is for null mutated mice, the so-called "knock out" mice.

In this review, recent progress on somatic cell gene transfer techniques and their application to nephrology are discussed.

STRATEGIES FOR GENE TRANSFER

Strategies of gene transfer techniques are classified into two categories: viral vector and non-viral vector methods. For human gene therapy, 75% of clinical trials thus far have been performed

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with viral vectors. Generally viral vectors afford a good transfection efficiency compared with the non-viral method.

Viral vectors

Retrovirus. Retroviruses have been employed in more than 50% of clinical trials [2] because the basic biology of the retrovirus has been extensively studied [6]. This virus binds to a specific receptor of the cells and transfers the viral RNA genome into the cytoplasm. Reverse transcription of the RNA yields proviral DNA, and the proviral DNA is transported to the nucleus where it is converted to a double stranded DNA and randomly integrated into the host genome. The stable incorporation of the expression cassette into the genome is advantageous for the potential long-term expression of the transgene. The major disadvantage of the retrovirus vector is the prerequisite that target cells must replicate to integrate into the host genome. Since many target cells in the kidney are non-dividing and terminally differentiated, retroviral vector-based gene transfer systems have restricted their use almost entirely to *ex vivo* applications in human gene therapy. To overcome this problem, a psudotype vector has been developed [15]. A retrovirus system was developed using two viral phenotypes [16]: HIV and vesicular stomatitis virus (VSV). Three plasmids were constructed for the system. The recombinant HIV was utilized for the expression cassette of the exogenous gene and the packaging construct. The plasmid coding cDNA for the G glycoprotein of vesicular stomatitis virus (VSVG) provided the envelope protein. The HIV based vector can transduce genes efficiently into non-proliferative cells *in vivo*. A potential risk of using the retrovirus is carcinogenesis. Random integration could potentially cause insertional mutagenesis by activation of oncogenes and disruption of tumor suppresser genes.

Adenovirus vectors. Recombinant adenoviruses have been used for gene delivery vectors. Native adenovirus is a double stranded DNA virus whose gene expression is controlled by four early regions and five late regions in the virus genome. The recombinant adenoviral vectors are deleted from the first early region (E1), which codes most of the functional proteins, including the transactivator protein of this virus. Thus, the recombinant adenovirus theoretically does not replicate by itself. The adenoviral vector has distinct advantages of high titers and is expected to show the highest level of expression of the transgene among the available vectors. The adenovirus vector has a significant advantage in delivering relatively large genes (up to 10 kb) into quiescent or terminally differentiated cells, since this virus can infect both dividing and non-dividing cells. However, the expression of the transfected gene is limited to weeks or months because the adenovirus does not integrate into the host cell genome. A crucial obstacle of adenoviral gene transfer is to elicit the appropriate immunological responses. The host response consists of an initial nonspecific inflammation followed by specific cellular and humoral immune responses directed at the cytotoxic T-lymphocyte (CTL)-mediated clearance of the recipient cells [17]. Therefore, the adenovirus vector cannot be employed for repeated gene transfer. In attempting to create a harmless adenoviral vector, a second generation adenovirus was produced that inhibited the transactivation of the major late regions by insertion of a temperature sensitive mutation into the E2 region [18]. Thereby, the second generation adenovirus showed prolonged expression of the transgene according to a reduced cytotoxic T-lymphocyte response. Third generation adenovirus vectors have been constructed to reduce the immunogenicity of the virus by further deletion of the E2a and E4 regions [19, 20]. These new vectors are expected to improve the toxicity profile and prolong the transgene expression by reducing the CTL-mediated elimination of transfected cells. However, this modification may result in the reduction of the titer of the modified adenoviral vector. Dematteo et al [21] reported another strategy to avoid the potent immune response to adenoviral vectors. They inoculated a recombinant adenovirus in the thymus of neonatal mice. When the virus was administrated intravenously to these mice in adulthood, they observed an impaired response of T cells against the adenovirus. The transfected adenovirus continuously expressed the transferred gene for up to 260 days.

Adeno-associated virus. Recombinant adeno-associated virus (AAV) have emerged as attractive alternatives to retroviral vectors. The AAV, a parvovirus, is a single stranded DNA virus that has the ability to integrate site-specifically on human chromosome 19 [4]. This virus can infect both non-dividing cells and dividing cells. However, a recent study demonstrated that the recombinant AAV does not always integrate on the chromosome 19 [22]. Despite that study, the generation of a recombinant virus is generally intricate and results in low viral titers. Baudard et al [23] reported a new method that uses the liposome as a vehicle for *in vivo* delivery of AAV vectors. The human multidrug resistance protein was successfully expressed in the spleen, liver and kidney by a single intravenous administration of a liposome-AAV complex. Kessler et al [24] demonstrated an attractive application of the AAV vector for gene therapy targeted to skeletal muscle. The sustained expression of the transgene for 32 weeks and the systemic delivery of biologically significant levels of a therapeutic protein for up to 40 weeks were observed after a single intramuscular administration of the AAV virus. Similar observations were reported on the long-term expression of the transgene by skeletal muscle-targeted AAV gene transfer [25].

Non-viral vectors

Liposome-mediated gene transfer. There are two concepts of liposome-mediated gene delivery [26]: the electrostatic type and the internal type. The electrostatic type of liposome, which was originally developed by Felgner et al [27], is made by the attachment of small cationic liposomes to negatively charged DNA. Cationic lipids are capable of forming positively charged liposomes that have been applied to *in vitro* gene delivery for more than 10 years because of their convenience and efficacy [28]. The composition of synthetic lipid is N-[1-(2,3-dioleyloxy]propyl]-N,N,N-trimethylammonium chloride (DOTMA). This electrostatic DNA-liposome complex is taken up into the cell mainly by phagocytosis. In contrast, the internal type of cationic liposome contains genetic materials inside the lipid bilayer. Theoretically, the liposome has no limitation on the size of the packaged genetic materials. The weakness of liposome-mediated gene transfer is that the expression efficiency is considerably lower than those obtained with viral vectors. Recently, the improvement of the transfection efficiency was reported by using a new cationic lipid in the formulation of liposomes. Goyal and Huang [29] developed the new cationic liposome containing 3β[N-(N′-N′-dimethylaminoethane)-carbamoyl]cholesterol (DC)-cholesterol, which is the only material that has been approved for use in human gene therapy. Wheeler et al [30] developed a new cationic liposome

composed of N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(dodecyloxy)-1-propaniminium bromide/dioleoyl phosphatidylethanolamine (GAP-DLRIE/DOPE). Lee et al also produced a new formulation [31]. These new cationic liposomes allowed significant improvement of the efficacy of *in vivo* gene transfer (1000- to 100,000-fold increase in expression). Another disadvantage of the present liposome-mediated gene transfer system is that it cannot target specific cells.

HVJ-liposome. The fusogenic character of HVJ, which belongs to the paramyxovirus family and has HN and F glycoproteins on its envelope, has been applied to HVJ-liposome-mediated gene transfer [32]. The HN and F glycoproteins cooperate to achieve a fusion of the virus and cell [33]. Whereas the HN protein binds to a receptor, sialic acid, and degrades the receptor as a consequence of its own neuraminidase activity, the F glycoprotein is cleaved to generate a hydrophobic fusion peptide by various proteases. The activated F glycoprotein can fuse with almost all of the cells except the peripheral lymphocytes. Native viral RNA and protein are introduced into the cytosol of the fused cell. The HVJ-liposome is classified as an internal type of liposome, and the HVJ-liposome method relies on this characteristic in combination with liposomes consisting of a lipid bilayer that can be used to encapsulate particular genetic material and proteins. The high mobility group (HMG)-1, a non-histone nuclear protein, undergoes nuclear translocation. Co-introduction of HMG-1 with DNA accelerates gene expression by several mechanisms including the facilitation of the translocation of the DNA into the nucleus [33].

The HVJ-liposome method possesses several advantages. (*1*) The HVJ-liposome method can effectively transfer DNA into undividing cells. (*2*) DNA is processed less by the endosome and lysosome systems in that DNA can be directly introduced into the cytoplasm and rapidly translocated to the nucleus by the HVJ-liposome method. (*3*) The HVJ-liposome method requires a relatively short incubation time (5 to 30 min). (*4*) The HVJ-liposome is capable of transferring double-stranded DNA at sizes up to 50 kb. (*5*) The HVJ-liposome transfection system has no apparent cytotoxic effects in small animals. (*6*) Repeated administration of the HVJ-liposome does not significantly affect the transfection efficiency, although the antibody against HVJ is produced. (*7*) Transfer of oligodeoxynucleotides (ODNs) has the particular advantage of long-term retention and stability of DNA in the nucleus by an unknown mechanism. The limitations of the HVJ-liposome method are as follows: (*1*) The gene expression is transient. (*2*) Targeting the delivery of the gene is impossible. (*3*) Preparation of the vector for the HVJ-liposome DNA complexes is complicated and the vector is less stable than in other methods.

New liposome method. Liposome can deliver genetic materials to a specific cell by conjugating with antibody or ligands. For example, liposomes combined with an antibody against a mouse major histocompatibility antigen could deliver DNA more efficiently to the target cells than the liposome-DNA complex alone [34]. The endothelial cells activated by IL-1β express E-selectin. Spragg et al achieved endothelial-selective delivery of DNA by conjugation of liposome with a monoclonal antibody against the extracellular domain of E-selectin [35]. The liposomes conjugated with the antibody bound selectively to activated endothelial cells. Fender et al recently reported a new device using a dodecahedron, that is, an adenoviral protein responsible for cell attachment, internalization and liberation of the virus into the cytoplasm [36]. They used the dodecahedron as a component of artificial cationic liposome system to improve the transfection efficiency. This idea may lead to a super-liposome containing specific adhesion proteins on its surface so that DNA can be delivered to the individual cells expressing the acceptor.

GENE TRANSFER INTO THE KIDNEY

The targeted gene transfer into the kidney has been a challenge since 1991 [37]. Strategies of gene transfer into the kidney are summarized in Table 1.

Targeting the glomerulus

The glomerulus is a focal point of the inflammatory response in the initiation and development of various glomerular diseases. Thus, the technique of gene transfer into glomeruli is attractive for understanding the mechanisms of glomerular diseases and may also be applied as a therapeutic approach to glomerular diseases.

In vivo gene transfer. There are at least two methods available for the *in vivo* gene transfer into the glomeruli: the HVJ-liposome method and a perfusion system of the adenoviral gene transfer. HVJ-liposome mediated gene transfer allows selective gene delivery to the glomeruli, mainly the mesangial cells [38, 39]. Fluorescein isothiocyanate (FITC)-labeled oligonucleotides (ODNs) were observed in almost all the glomeruli within 20 minutes after transfection, suggesting the susceptibility of HVJ-liposome mediated gene transfer to mesangial cells [38]. However, the mechanism of the selective delivery to mesangial cells is not well understood. Even with the high efficiency of the gene transfer, the expression of the gene was restricted to 15 to 35% of the glomeruli [40–42]. The intraglomerular expression of the transfected gene is limited to, at most, seven days. The critical issues of HVJ-liposome gene transfer are thus the low transfection efficiency and the short duration of the expression.

Generally, intra-arterial injection of adenovirus by itself, even with incubation by clamping the renal vessels, does not lead to successful gene transfer into kidney. Tryggavason and colleagues [43, 44] demonstrated for the first time the successful gene transfer into the glomerulus by means of the adenovirus vector. They tried to introduce the adenovirus vector by means of the kidney perfusion system, and perfusion of the adenovirus was performed for two hours. Four days later transgene expression was observed in 75% of the glomeruli and the expression lasted for three weeks. The expression of the transgene, β-galactosidase, was observed mainly in podocytes, while expression of β-galactosidase was weaker in endothelial cells and mesangial cells. Interestingly, they did not observe any expression of the transgene in parietal epithelial cells and tubular cells. The adenovirus perfusion system allows selective gene transfer targeting the glomerulus with high efficacy.

Ex vivo gene transfer. Two strategies have been reported on glomerular targeted *ex vivo* gene transfer: infusion of genetically-modified mesangial cells into the renal artery and transplantation of a kidney with an already transferred gene. This approach can avoid the contamination of the transfection accessories such as viral proteins or chemicals. Extensive work concerning *ex vivo* gene transfer using the mesangial cell vector system has been done by Kitamura et al [45, 46]. The established mesangial cell line continuously expressing the transfected foreign gene was injected into the renal artery. Mesangial cells were trapped in the capillaries of the glomeruli and the expression of the exogenous

Table 1. Strategies for gene transfer into the kidney

In vivo gene transfer into the kidney

Species	Targeted region/cell	Vector	Access	Duration of expression	Reference
rat	glomerulus	HVJ-liposome/plasmid	renal artery	7 days	[40, 41, 42]
pig	glomerulus	adenovirus	renal perfusion (2 hr)	3 weeks	[43]
rat	proximal tubule	native ODNs	peripheral vein	ND	[47, 48]
rat	proximal tubule	DOTMA;DOPE/native ODNs	peripheral vein	ND	[66]
rat	proximal tubule	DOTMA:DOPE/plasmid	renal artery	3 weeks	[50]
rat	proximal tubule	adenovirus	renal artery	1–2 weeks	[52]
rat	proximal tubule	retrovirus	renal artery after folate injury	3 weeks	[51]
rat	interstitium outer medulla	adenovirus	renal artery cold/vasodilater	4–8 weeks	[53]
rat	outer medulla	DOTMA:DOPE/plasmid	renal pelvis	6 week	[50]
rat	IMCD	adenovirus	renal pelvis	1–2 weeks	[52]

ex vivo gene transfer to the kidney

Species	Targeted region/cell	Vector cell	Access	Duration of expression	Reference
rat	glomerulus	mesangial cell	renal arterial	4–8 weeks	[45]
rat	glomerulus	macrophage	renal arterial	ND	[89]
mouse	interstitium subcapsular	proximal tubule cell	renal subcapsular	4 weeks	[56]
rat	glomerulus/tubule	metanephros	renal subcapsular	3 weeks	[37]
pig	glomerulus	adenovirus	renal perfusion (12 h)	2–3 weeks	[43]
human	proximal tubule	adenovirus/polylysine-Ab	perfusion	ND	[54]

ND is not determined.

gene was observed in the glomeruli for four weeks. This mesangial cell vector system provided a strong and long-term expression of the transfected genes *in vivo*. However, the transplanted mesangial cells did not migrate into the original mesangial area, but remained in the endocapillary lumen.

A kidney for transplantation could be a good target for gene transfer. Heikkla et al perfused isolated pig kidneys with a solution containing adenovirus. The perfusion of the adenovirus vector through the renal artery for 12 hours resulted in intense expression of the reporter gene in 85% of the glomeruli [43].

Targeting the tubule

The tubule is composed of more than ten different cells that play various roles in concert to maintain fluid and electrolyte homeostasis. Recent progress in molecular nephrology has revealed nephron heterogeneity based on the presence of functional components in each nephron segment, for example, various channels, transporters, enzymes, etc. Therefore, the correction of the nephron dysfunction should be accomplished by correction of a particular molecule in the individual nephron segment. However, there are no specific tubular cell-targeted gene transfer techniques. Obstacles regarding gene transfer in tubular cells include: (*1*) for an approach from the renal artery, genetic materials must pass through the glomerular basement membrane; and (*2*) selective delivery to the proximal segments is difficult using a retrograde approach.

The simplest transfer into the proximal tubule is the intravenous administration of genetic material. The intravenously injected oligonucleotides (ODNs) accumulate in the proximal tubule by phagocytosis. Rappaport et al [47] studied the fate of ^{32}P-labeled ODNs after intravenous injection. The labeled ODNs were predominantly localized in kidney and liver and were detected in the proximal tubular cells within 30 minutes after injection. Oberbauer, Schreiner and Meyer [48] also reported that intravenously injected ODNs gathered in proximal tubules, and electron microscopic observations showed that ODNs did not merely accumulate in the brush border or lysosomal compartment in proximal tubular cells, implying that they were not totally degenerated after being phagocytosed by the proximal tubule. Oberbauer et al [49] applied this characteristic of the ODNs that were taken up by the proximal tubular cells to inhibit a sodium/phosphate cotransporter (Na/Pi-2). A single injection of the antisense ODNs against the Na/Pi-2 cotransporter inhibited both mRNA and the protein for the Na/Pi cotransporter, and consequently suppressed phosphate uptake into the brush border membrane vesicles of the proximal tubule. These results suggest that the renal proximal tubule is a good target for antisense therapy.

Lien and Lan [50] reported that the DOTMA:DOPE-DNA complex can be successfully transferred into the tubular cells by intra-renal artery injection as well as by renal pelvic injection in mice. They observed the expression of the reporter gene in the tubule mainly in the outer medulla for six weeks after intra-renal pelvic injection. However, the expression of the inner medulla is weak and has no expression in the glomeruli or vascular or interstitial components. Intrarenal arterial injection of a DOTMA:DOPE/DNA complex successfully expressed the reporter gene in the tubular cells in the outer medulla and cortex two to three weeks after injection. However, no positive expression was observed in the glomeruli and expression of the transgene was not seen after intrarenal parenchymal injection. They applied this transfection to gene therapy for the carbonic anhydrase II deficient mouse [50]. In consequence of this study, in the mouse transfected with a carbonic anhydrase II gene there was a partial correction of the renal tubular acidosis.

The direct retroviral gene transfer to the proximal tubule was

reported by Bosch, Woolf and Fine [51]. Rats were pretreated with cytotoxic reagent folic acid to induce proliferation of the proximal tubule, and then retrovirus vector was injected into the renal artery. In consequence, a weak expression of the reporter gene was detected in the proximal tubule.

Recently, three different reports concerning *in vivo* adenoviral gene transfer to the kidney have been published. Moullier et al [52] observed very weak and patchy expression of the reporter gene in the cortex when the adenovirus were simply injected into the renal artery. The expression lasted for two weeks. The retrograde transfection from the renal pelvis caused intense expression of the reporter gene in the papilla and medulla. The expression lasted for one to two weeks. In contrast, Heikkila et al demonstrated that no tubular expression was observed by perfusing the kidney with the adenovirus [43]. In addition, Zhu et al reported no significant transfection of adenovirus to the tubular cells, but that there was transfection to the interstitial vasculature, as discussed below [53]. The reasons for these different outcomes despite using similar adenoviral vectors needs to be clarified.

Zeigler et al reported the first trial of *ex vivo* gene transfer into the isolated human kidney under the conditions of organ preservation [54]. They used an adenovirus polylysine DNA complex and pulsatile perfusion for two hours at 4°C. The gene delivery and expression were localized to a significant fraction in the proximal tubular epithelial cells.

Targeting the interstitium

Zhu et al reported on a successful adenoviral vector transfection into the interstitial compartment [53]. Their method for gene transfer to the kidney is cold incubation and use of vasodilators. They injected an adenovirus vector solution containing papaverine into the renal artery of a cold kidney packed with frozen phosphate buffer saline, and both renal artery and vein clamped for 45 minutes. The prominent expression of the reporter gene of β-galactosidese was observed in the interstitial vasculature including arterial blood vessels in outer medulla in both the outer and inner stripes and in periglomerular and peritubular capillaries in cortex, but not in tubular epithelial cells. They also challenged the cold preservation technique for gene transfer to a rat model of polycystic kidney disease (Han:SPRD rat). Adenovirus was infused into the renal pelvis followed by a 30 to 60 minute incubation. The β-galactosidase activity was seen in cortical interstitial tissues including interstitial cells and epithelial cells of the cyst in the Han:SPRD rat.

As an *ex vivo* approach, the implantation of genetically-modified tubular epithelial cells (TEC) into the subcapsular region has been reported [55, 56]. The transplanted TEC remained under the renal capsule and the transferred gene maintained its expression for at least four weeks. The authors applied this modified TEC as a vehicle to deliver a selected cytokine to examine whether a specific molecule causes the tubulointerstitial injury.

STRATEGIES OF GENE THERAPY FOR EXPERIMENTAL RENAL DISEASES

Antisense therapy

The ability of short, single stranded ODNs to interdict individual gene expression in a sequence specific manner is the basis for antisense therapy [57–60]. Antisense ODNs, which are designed to bind to complementary mRNA, can inhibit the gene expression by cleavage of the target mRNA with RNaseH and/or by attenuation of translation. Antisense ODNs, usually 15 to 25 nucleic acid bases long, are designed to use a unique sequence relative to the entire genome and to avoid simple repeated or palindrome sequence. The translation initiation site is often used for antisense design. The other sequence from the 3′ non-coding region, however, may confer the strongest inhibitory effect [61]. Antisense ODNs are modified by phosphorothioate linkage to stabilize the ODNs against nucleases. Although the antisense effect is based on the Watson-Crick base pairment formation, one should be cautious with the effect of non-antisense ODNs. Therefore, it is essential to demonstrate that the antisense effect is comparable with the reduction of the targeted mRNA as well as the protein level. Burgess et al [62] reported that four consecutive guanosine residues inhibit cell proliferation *in vitro*. A sequence independent induction of transcription factor Sp1 by phosphorothioate ODNs was also reported [63]. There are two reports concerning the antisense effect on the progression of glomerulonephritis. Both of them used the internal type of liposomes to target the glomeruli. Akagi et al [38] demonstrated that anti-TGF-β antisense ODNs inhibited the TGF-β expression in Thy1 glomerulonephritis using the HVJ-liposome method. They transferred the ODNs selectively to glomeruli two days after injection of anti-Thy1 antigen. Glomerular ECM accumulation was significantly suppressed in nephritic rats in parallel with the reduction of TGF-β expression in the glomeruli. Kashihara, Maeshima and Makino [64] reported a similar effect on the suppression of ECM accumulation using antisense ODNs for TGF-β. They also demonstrated that treatment with platelet-derived growth factor-B (PDGF-B) antisense ODNs suppressed hypercellularity in the glomeruli of Thy1 glomerulonephritis. Alternatively, proximal tubule targeted antisense therapy was reported. Intravenous injection of antisense ODNs resulted in their accumulation in the proximal tubule and inhibited individual gene expression. Noiri et al [65] applied antisense ODNs against inducible nitric oxide (NO) synthase in the inhibition of inducible nitric oxide synthase (iNOS) in an ischemic kidney. A single intravenous injection of iNOS antisense ODNs attenuated acute renal failure and improved the morphological changes. Haller et al [66] reported attenuation of ishemic-reperfusion injury by administration of antisense ODNs against ICAM-1. They transferred ODNs with DOTMA:DOPE six hours prior to 30 minutes of bilateral artery clamping. The ICAM-1 expression was significantly suppressed by antisense treatment. Consequently, the cortical renal damage and leukocyte infiltration was attenuated in association with preservation of renal function. Antisense ODNs are potentially applicable for the intervention of various renal diseases, although the short-term effects and targeted delivery give reasons for concern.

Transcription factor decoy

A novel gene therapy strategy using a transcription factor decoy has been reported by Morishita et al [67]. Double stranded ODNs containing a *cis*-element for a particular transcription factor act as a "decoy." The transfection of an excess decoy using an internal type liposome results in the attenuation of the authentic *cis-trans* interaction of the gene promoter leading to the dissociation of the transcription factor from the endogenous *cis*-element. Consequently, the particular gene expression is inhibited. Decoy strategy has some advantages over antisense strategy: (*1*) double stranded DNA is more stable, (*2*) a single decoy can suppress

multiple genes coding of the same *cis*-element in the promoter region, and (*3*) a decoy can inhibit the constitutively expressing factor. Two reports have come out on the inhibitory effects of the progression of Thy-1 glomerulonephritis. Both the E2F decoy [68] and the NFκB decoy [64] successfully inhibited the mesangial cell proliferation and ECM expansion in experimental glomerulonephritis. The disadvantage of decoy strategy is its inability to selectively inhibit single gene expression. The genes coding a targeted *cis*-element in promoter region may eventually be suppressed by the decoy ODNs.

Muscle targeted gene therapy

Systemic delivery of the therapeutic protein made by the transferred gene is an alternative method of gene therapy [69]. Skeletal muscle is the target of gene therapy because the muscle is easily transfected, there is an abundance of capacity and it is highly vascularized. After gene transfer, the transgene is able to produce secreted protein constantly for two to three weeks [70, 71]. Producing an expression vector system is less expensive and overcomes the need to highly purify a recombinant protein. The long-term expression of the transgene and stable delivery of the produced protein by means of skeletal muscle gene transfer may provide a practical strategy for the treatment of inherited and acquired protein deficiencies. Isaka et al applied decorin gene therapy to the treatment of experimental glomerulonephritis [72]. The proteoglycan decorin is a natural inhibitor of transforming growth factor beta (TGF-β). They transferred a plasmid vector expressing decorin into rat skeletal muscle by the HVJ-liposome method, and confirmed the increase in production of decorin in the skeletal muscle and the accumulation in the kidney. The glomerular extracellular matrix (ECM) accumulation and proteinuria were significantly suppressed in transfected nephritic rats in comparison with the reduction of TGF-β mRNA and its protein. This result suggests that manipulation of TGF-β gene expression in the process of glomerular injury may ameliorate the progression of glomerulonephritis. To this end, Isaka et al also constructed artificial soluble TGF-β type II receptors that are composed of the extracellular domain of the TGF-β type II receptor and IgG Fc [72]. The chimeric soluble receptor inhibits both TGF-β actions on cell proliferation and ECM metabolism in cultured cells. In addition, the chimeric protein that was systematically supplied from the expression vector significantly inhibited ECM accumulation in the glomeruli of animals with experimental glomerulonephritis.

The adenovirus was also used for skeletal muscle gene transfer [73, 74]. The adeno-associated virus (AAV) vector was reported to express the transgene in skeletal muscle and to last the systemic delivery. Kessler et al reported that after a single intramuscular administration of an AAV vector containing a gene for human erythropoietin, it was expressed in the muscle for 40 weeks [24]. In consequence, the serum level of erythropoietin reached 700 mU/ml and the hematocrit was sustained over 80%. Gene therapy transferring AAV vectors into the skeletal muscle may be feasible to provide systemic supplementation of the individual protein for the treatment of inherited and acquired protein deficiencies.

Mesangial cell vector system

As mentioned above, the mesangial vector system is a sophisticated approach to examine the action of a specific molecule in glomeruli. Kitamura et al established mesangial cell line expressing TGF-β continuously and injected to the renal artery to deliver the TGF-β expressing mesangial cells to the glomerulus [75]. In consequence, [^{3}H] thymidine incorporation into glomerular cells was suppressed, suggesting that TGF-β produced by exogenous mesangial cells inhibits the [^{3}H] thymidine incorporation into glomerular cells. Furthermore, they observed a similar inhibition of the suppression of mitogenic response in nephritic glomeruli induced by anti-mesangial cell antibody. However, it is possible that the long-term existence of exogenous mesangial cells may elicit the immunological reaction.

FUTURE PERSPECTIVES

Control of the expression of transfected genes

If the transferred gene is continuously expressed after a successful intervention of the disease, there must be a mechanism for switching it off. An ideal gene transfer system should possess the regulator since the transfected gene may overproduce the coding product in some situations. For this purpose, several approaches have been reported using inducible eukaryotic promoter systems responding to heat shock, heavy metals, steroid hormones, cytokines and aromatic hydrocarbones. These systems are unfulfilled as a functional molecular switch, since they are a less sensitive on/off system. Gossen and Bujard developed a sophisticated regulatory system using a tetracycline-regulated transactivation system [76]. This method is based on the regulatory element of the tetracycline resistance operon of *E. coli*, in which the transcription of resistance-mediating gene is negatively regulated by the tetracycline repressor (tetR). In the presence of tetracycline, tetR does not bind to its operators located within the promoter region of the operon. In this system, a tetracycline-controlled hybrid transactivator (tTA; a fusion protein between the tetracycline repressor of the *E. coli*-derived operon and the activating domain of the herpes simplex virus protein, VP16) can induce the transactivation of the exogenous promoter containing the tetracycline operetor sequences in the absence of the tetracycline. Tetracycline inhibits this tTA-dependent transactivation by binding to tTA. Kitamura applied this system to the mesangial cell vector system [77]. Mesangial cells were co-transfected with a regulatory plasmid coding tTA and a response plasmid coding LacZ driven by promoter containing tetracycline operator sequences. The established mesangial cells were transplanted in the glomeruli via the renal artery. When tetracycline was withdrawn the expression of β-galactosidase was observed in three days, and in turn, oral administration of tetracycline completely suppressed the expression of β-galactosidase in three days. The tetracycline regulatory system is strict, reversible and site-specific control over transgene expression in the glomerulus. This regulatory system provided a suitable control system for adjusting the delivery of therapeutic proteins for more than 20 weeks in mice transplanted with engineered primary myoblasts [78]. No, Yao and Evans reported an insect steroid hormone, the ecdysone regulatory system [79]. They produced two lines of transgenic mice, one carrying a modified ecdysone receptor gene together with retinoid X receptor (RXR) gene and the other carrying an ecdysone-responsive promoter ligated to a reporter gene. They obtained a hybrid mouse carrying both genes, as shown by bleeding, and observed the ecdysone induction of the reporter gene. In consequence, they reported that this system provides a lower level of basal activity and has higher inducibility (induction reaching 10,000-fold higher

in magnitude) compared with the tetracycline system. Delort and Capecchi [80] also reported a similar system using a synthetic steroid receptor (TAXI), which binds antiprogestins, such as mifepristone (RU486) and an inducible promoter (UAS) containing an RU486 responsive element.

Cre recombinase has been used for the control of the activation/inactivation of the transgene in the genome [81]. Cre binds to a 34 nt loxP site and mediates the excisional deletion of a DNA sequence flanked by a pair of loxP sites. The gene activation can be achieved by the excisional deletion of the stuffer DNA that lies between the promoter and coding region. In turn, the strategy of the gene inactivation can be completed by deletion of the enhancer element from the promoter region. The Cre-LoxP system is applied to the transgenic mouse carrying two LoxP sites in targeting the promoter, which turns the molecular switch on by the infection of the adenovirus vector coding Cre gene [82–84]. The obstacle to this Cre-LoxP system is that the molecular switch causes irreversible changes in the transgene.

Transcriptionally targeted gene therapy

It is important to restrict the expression of the transferred gene to the individual cells. Cell-specific or tissue-specific gene expression is regulated by a *cis*-acting control sequence and *trans*-acting factor. Kidney specific promoter may be useful if the gene delivery system cannot target to the renal cell. The expression vector cassette driven by the viral promoter and kidney specific enhancer may select the gene expression in the renal cells. Miller and Whelan reviewed tissue specific genes and their specificities [85]. To use the *cis*-element in the particular promoter of expression vector may allow the cell specific expression *in vivo*. The phosphoenolpyruvate carboxykinase gene [86], the Na-K-2Cl transporter gene [87] and aquaporin 2 gene [88] are reported to show nephron segment-selective gene expression. However, the cellular sequence is sometimes overridden by the strong viral transcriptional controls in the viral based expression cassette.

Tissue and cell engineering

Transplanted kidneys have become a new target of gene therapy. The gene transfer technique may apply as an interventional strategy to inhibit mediators of the immunologic response in rejection of the transplanted kidney. As mentioned above, Zeigler et al aimed to reduce the rejection of a transplanted kidney by using the immunosuppression of an isolated organ [54]. Another method is *ex vivo* gene transfer to renal cells or hematopoietic cells. Kitamura et al have developed the mesangial cell vector system [45] and macrophage vector [89]. Cells that were genetically modified and given a specific function *in vitro* were injected into the renal artery to be delivered to the glomerulus. This method may be potentially applicable as a novel therapy to provide the immunosuppressive proteins and antifibrotic molecules at the inflammatory glomerulus *in situ* [90]. The transplantation of the tissues containing genetically modified myoblasts in silicone rubber molds is a new strategy for gene therapy [91]. Subcutaneous organoid implantation allows systemic delivery of a bioactive compound and subsequent surgical removal leads to its rapid disappearance. Hume has proposed a concept of the tissue engineering of a bioartificial kidney that will be synthesized with genetically modified cells and hollow fibers [92].

CONCLUSION

We still have no satisfactory and reliable gene transfer technique for the kidney. A more effective and selective gene transfer technique needs to be developed for human gene therapy as well as for research to understand the pathophysiology of renal diseases. The ambition of understanding the molecular mechanisms of the kidney in physiological and pathological conditions, and enthusiasm to correct the cause of diseases at the fundamental level must become strong driving forces to develop new technologies to overcome the current obstacles in gene transfer.

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APPENDIX

Abbreviations used in this article are: ODNs, oligonucleotides; ADA, adenosine deaminase; VSV, vesicular stomatitis virus; VSVG, G glycoprotein of the VSV; HIV, human immunodefficiency virus; E1, early region in the virus genome; CTL, cytotoxic T-lymphocyte; AAV, adeno-associated virus; DOTMA, N-[1-(2,3-dioleyloxy]propyl]-N,N,N-trimethylammonium chloride; DC-cholesterol, new cationic liposome containing 3β[N-(N′-N′-dimethylaminoethane)-carbamoyl]cholesterol; GAP-DLRIE/DOPE, N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(dodecyloxy)-1-propaniminium bromide/dioleoyl phosphatidylethanolamine; TGF-β, transforming growth factor-beta; ECM, extracellular matrix; PDGF-B, platelet-derived growth factor-B; NO, nitric oxide; iNOS, inducible nitric oxide synthase.

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