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REMEDIAL APPLICATIONS OF SILENCING RIBONUCLEIC ACIDS AND MODALITIES FOR ITS DELIVERY TO THE KIDNEYS - A REVIEW

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Abstract

Background: The Kidney has been the target organ for the delivery of silencing ribonucleic acids (silencing RNA) administered systemically in comparison to other body tissues.
Materials and method: In this review, we discussed different approaches made to delivering proteins to the kidneys in different conditions like normal and pathological defects. Data from clinical experiments have been used to discuss and support the administration of silencing RNA for the treatment of kidney diseases.
Results: Results were achieved using the available genome wide RNA libraries.
Conclusion: The research results are helpful in application to 3D and conventional models to find the involvement of signal pathways in kidney diseases.

Key words: silencing RNA, kidney disease, targeting proteins, signal pathways.

Introduction

The discovery of ribonucleic acids interference (RNAi) had been given more attention in gene transfer and used as experimental tool in biological and molecular research (Racz and Hamar, 2006). RNAi In this technique the double stranded RNA is used in the sequencing of mRNA which inhibits the translation and target proteins lower expression. The libraries of silencing ribonucleic acids (silencing RNA) are best source/s for the identity and disease targeting (Kim et al., 2005). Silencing RNA are used in the therapy of different clinical disease like kidney transplant, diabetic nephropathy primary glomerular diseases (Chen et al., 2008), and mostly in the treatment of viral infection and tumors (Schmitt et al., 2008). Kidney is the best target site due to the rapid uptake of silencing RNA and resulting lowering of targeted proteins expression, which is an indication for further analysis in renal disease treatment (Budker et al., 2006).

Basic advantage of silencing RNA is that it does not affect the influencing pathways but act on pathways associated with disease signaling. This advantage is helpful in preventing the existing injury and not creating new injury in the kidney. In recent studies, focus is on the potential of RNAi to treat renal diseases and injuries in kidneys verified in experimental animals used as model.

For a better understanding of RNAi an attempt must be made to understand its mechanism of action. Ribonuclease is used to cleave the double stranded RNA into small pieces of 21-23 nucleotides. These fragments are then bounded to RNA induced silencing complex (RISC), Argonaute-2, which separate and unwinds both strands of silencing RNA. The RISC then starts its degradation of mRNA selectively (Hamar et al., 2004). The RISC after mRNA cleavage again in a new series start cleavage of the new mRNA, which prevent synthesis of proteins and does not cause any permanent effect on the genome. This process continues from several days to several weeks (Zheng et al., 2009).

The effects of silencing RNA were studied *in vivo* and found that the proteins expression was low due to degradation (Takabatake et al., 2005). Plasmid DNA having sense 21 and antisnse nucleotide are helpful in the formation of short hairpin RNA. Procedure for this is by separating sense and antisense sequence by non-coding loop. Self binding of complementary nucleotide sequence and folding of RNA lead to the formation of regular shRNA while loop domain cleavage result in the formation of regular silencing RNA. Synthetic silencing RNA are then directly loaded on to RISC after injection into cells. Injection of silencing RNA into the cells results in the dicer processing and that was less potent than 25-27 nucleotides (Xia et al., 2008). The off target effect of silencing RNA studied are induction of inflammatory response, activating anti-angiogenetic response or regulation of non-targeted proteins (Wesche-Soldato et al., 2005). These effects are thought due to the immunogenic response produced by the body to the recognition of double stranded DNA as foreign object or, due to the presence of seed sequence in silencing RNA (Jere et al., 2009).

Modalities for delivering silencing RNA to kidney

Effective knockdown at the target site has been a manor concern of using silencing RNA techniques *in vivo*. Expected non-specific or off-target side effects may affect the expected results and outcome. Silencing RNA application in local drug delivery have been effective while system delivery still needed more work as difficulties are faced in this at the present time. In most systemic administration the kidney is preferred along with spleen and liver. It is selected due to the reason that the absorption of silencing RNA by nephron and excretion from the body by kidney have major role. Exogenous nucleotides or nucleotide complex are administered in the body by Intravenous administration. The efficient and easy way for administration of plasmid DNA into the body is using hydrodynamic injection in to the tail vein (Malek et al., 2009). Injection of silencing RNA by IV route has been used different varieties of studies, which showed variation in renal targeting rates due to transfection agents and has been described to conclude in changing the rates of renal targeting, on the basis of the use of transfection reagents. Application of

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150 µl (100 µg) single dose of silencing RNA administered systemically for specific multidrug resistance protein isoform 2 (Mrp2) distributed rapidly to the kidney and showed good result in lowering the Mrp2 activities after 4 days treatment in treating proximal tubules. Radiolabelled silencing RNA when administered showed 3 times more concentration in the kidney of silencing RNA (Budker et al., 2006).

The role of Zag proteins was studied in animal models using mouse for treating ischemia-reperfusion injury, the solution of silencing RNA in saline were diluted and 200 µl were injected daily for duration of 3-7 days using retro-orbital sinus, resulted in the knockdown of renal protein (Xia et al., 2009). Large volume of solution containing silencing RNA was required for the hydrodynamic delivery approach by I.V. route. An injection volume of 10% of body weight was used for hydrodynamic delivery, in the original protocol for duration of (15-30 s); whereas 50-100 µl was the generally accepted average for IV injections in mouse tail veins. These results showed a hypervolemia causing transient capillary dysfunction and even leakage, which may cause parenchymal tissues cells exposure to silencing RNA. It was found that hydrodynamic delivery in most cases lead to liver damage (Krebs et al., 2009). Results of this approach have potential for access to the kidney, silencing RNA availability in tubular lumen and peritubular interstitium access from the basolateral side monolayer.

Fast uptake of silencing RNA was observed from the tubular lumen in hydrodynamic approach in different models of mouse in different studies. Dilution of silencing RNA in BPS in volume of 1 ml resulted in flush after an alternated administration of 0.3 ml of BPS for 24-36 hours and protein knockdown in hydrodynamic delivery (Kinoshita et al., 2005). Tissues containing silencing RNA may be used in the possible heart transplantation in animal models (Negishi et al., 2008). In comparative studies it was observed that silencing RNA labeled with fluorescent administered rectal and intra-peritoneal significant uptake was observed in the rectal administration after 4 hours of the dosing (Shukla et al., 2009). This study showed that silencing RNA was useful in tissue retaining but proteins were not studied. It also showed that application of high pressure to the kidney may result in kidney dysfunction due to overload of silencing RNA (Geary et al., 2009).

Different routes may be used for the intrarenal and local delivery i.e. targeting gulmeruli using renal artery (Anders et al., 2004), targeting the tubule-interstitium via renal vein (Kinoshita et al., 2005), renal pelvis via intrauretral administration (Robbins et al., 2009) and for intraparenchymal silencing subcapsular administration. *In vivo* electroporation enhances silencing RNA delivery and enhanced administration of transgenic construct intra-arterial in rats which resulted in mesengial cells expression (Kim et al., 2007). Mild ischemia was observed when administration via renal artery was performed, that cause occlusion of artery temporarily. This condition decreased susceptibility to ischemic insult (Nyengaard et al., 2009) and affected the experimental results. Transgeinc expression was observed with intrapelvic injection in the medulla of kidney which affected the tubular epithelium but no such effects were observed in the inner region of medulla. Intraurethral administration of DNA enzyme resulted in the transgene expression in the interstitial cells followed by kidney electroporation (Mook et al., 2007). Invasive techniques are required for the subcapsular administration of renal capsule and parenchyma cells which caused minor renal dysfunction. Some part of the volume administered will be lost through the puncture hole after needle retraction, which limit the application of this approach.

Systemic administration of the unmodified, naked and insufficient silencing RNA resulted in the depletion of targeted proteins. It was observed in GFP-transgenic mice that silencing RNA administration resulted in moderate reduction in renal GFP expression (Kennedy et al., 2004). This effect was observed due to the degradation of silencing RNA by endo- or exonucleases (Takabatake et al., 2005). Cells uptake of silencing RNA other than additional carrier may be possible but is less efficient in comparison to transfection reagents which encapsulate or form complex with silencing RNA (Schmitt et al., 2008). Application of silencing RNA's libraries was necessary for pre-synthesized silencing RNA administration. It was observed in several studies that uptake of silencing RNAs by tubular epithelium was enhanced by the application of lipid-based cationic trasfection agents. For delivery of biochemical agents, liposomes were widely accepted source. For delivery of silencing RNA to kidney, N-[1-(2,3-Dioleoyloxy) propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP) was used (Takahashi et al., 2009). DOPC application as carrier for silencing RNA was found to enhance the uptake of silencing RNA by lungs, liver and kidney tubules. This resulted in the protein knockdown by kidney epithelium (Nakamura et al., 2004). For enhance efficacy of silencing RNA, novel agents i.e. lipectamine have been developed. For studies, complex of lipofecatmine and silencing RNA was formed and injected through renal vein in 300 µl volume, which showed silencing RNA uptake by tubular cells in 20-30 minutes.

Polyethylenimine complex with silencing RNA not only enhanced the uptake but also prevented degradation of silencing RNA. It also facilitates the release of silencing RNA into the cytoplasm (Ortiz et al., 2003). It was observed in studies that naked silencing RNA was mostly degraded in kidney while complex with PEI was available in the kidney tubules and not degraded (Seregin et al., 2009). The important benefit of PEI-silencing RNA complex is its endosomal disruption, which result in the silencing RNA in cytoplasm (Grimm and Kay, 2007). Another approach for delivery of silencing RNA is PEG-POLY-L-LYSINE copolymer as nanocarrier. This complex formation enhanced the silencing RNA uptake by kidney as compared to the studies performed using pseudovirion vector as carrier (Schmitt et al., 2008). Antibody mediated targeting is another approach for cell specific delivery of silencing RNA. The strategy followed was complex formation of IgG with neutravidin, biotinylated proatime and silencing RNA. Analysis resulted that antibody was bound to podocytes not to tubular cells, which may result in glomerulo-sclerotic injury. This approach resulted in specific targeted proteins knockdown. The antibodies were also detected in colon tissues, spleen, liver, lung and muscles (Wang et al., 2006). Complex of silencing RNA to streptavidin conjugated antibodies was developed as alternative to protamine-silencing RNA complex, PEGylated liposomes as carrier for silencing RNA and antibodies conjugation with cationic lipid-silencing RNA complex (Shou et al., 2009). The new approach is application of biodegradable hydrogels for silencing RNA delivery (Zheng et al., 2008). These hydrogels are used for delivery of plasmid DNA in extended release form as well carrier for delivery of silencing RNA (Molitoris et al., 2009). For delivery of silencing RNA into skin and muscles, electroporation techniques have been used (Kushibiki et al., 2006). As technique for delivery of silencing RNA in rates, electroporation have enhanced silencing RNA uptake and knockdown of targeted proteins in glomeruli (Chen et al., 2008). But electroporation induce stress signaling of cells (Luo et al., 2008). Alternative for this is sonoporation to enhance silencing RNA uptake (Hoffman et al., 2010). The other technique used was ultrasound enhanced nucleotide, which was useful in silencing RNA delivery to skin, which was not effective in kidney (Krausz et al., 2007).

The destabilization of naked silencing RNA is the major problem due to sensitivity of phosphodiester linkages to nucleases in cells as well serum. Chemically modified silencing RNA have better stability and improvement in renal targeting (Antczak et al., 2009). Chemical modification of phosphodiesters help in the protection from the serum nucleases and exonucleasese (Masszi et al., 2004), which is due to replacement of non bridging oxygen and prolong half life (Racz and Hamar, 2006). Phosphorotthionate *in vitro* studies shown low toxicity and high distribution in systemic delivery but have toxicity in renal system due to some specific proteins (Masszi et al., 2004). Linkage of Phosphothoate with silencing RNA has reduced degradation by enzymes and more accumulation in bone marrow, kidney and skin (Antczak et al., 2009). Complexing with lipids results in the increased uptake by cells as well increase in half life (Krebs et al., 2009) and accumulation in certain organs in mice.

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Tubular epithelium express Toll Like Receptor (TLR) which bind to RNA and function as signal for the possible viral infection (Shou et al., 2009), which activate immune response as well degradation by endonucleases (Xia et al., 2009). Modification of silencing RNA was studied and it was found that modified form was more resistant to nucleases but activated immune system (Nakamura et al., 2004).

PEG complexing was found helpful in reducing immunogenic response but also have increased stability against nucleases as it is less toxic and more soluble (Takahashi et al., 2009). Its half life was increased as well as increase was observed with PEGylation. PEGylation makes the molecules more hydrophobic as well reduce immunogenic effects produced. PEGylation was found helpful in micelle formation and targeted drug delivery to receptors (Hoffman et al., 2010).

Another class of nucleic acids called Locked Nucleic Acids have sugar phosphate backbone, easy to synthesized, soluble in aqueous media and able to combine with RNA, DNA to form duplex with specific RNAs. LNA studies showed increase to degradation by serum nucleases and expression of target proteins (Anders et al., 2004).

Nonionic DNA analogue, called morpholino oligonucleotides, was resistant to digestion by nucleases and not a substrate for RNase and block GFP expression in zebrafish embryos (Asad et al., 2013). It blocks maturation of micro RNA and its activities but have prolonged activities at low concentration in comparison to unmodified silencing RNA. Modification of MO and LNA to silencing RNA has more stability *in vivo* with reduction in off target effects. Chemically modified silencing RNA has been studied for targeting kidney and was found that celegans enhance silencing RNA cleavage by exonucleases (Masszi et al., 2004).

Vectors containing shRNA have been used as alternative to silencing RNA in employing plasmid DNA vectors in kidney (Anders et al., 2004). This has been used for delivery of nucleus to participate in transcription. Vectors of pDNA are more time-consuming compared to silencing RNA synthesis and long term strategies are required. Viral vectors are more advantageous due to stability and are more efficient (Budker et al., 2006). Delivery of adenovirus in rats shown transgenic expression in glomeruli, tubules as well medullary epithelium but sever adverse effects limit its application (Liu et al., 2013).

Vectors of adeno- associated virus (AAV) studies showed different behavior from adenoviral vector in transgenic expression and life cycle (Waqas et al., 2013), activation of immunogenicity (Luo et al., 2008) and tissue specification (Zhang et al., 2013). During studies, recombinant AAV in epithelium showed transgene expression, but showed transgene expression in kidney medullary area after intrapelvic delivery (Antczak et al., 2009). It was found that AAV-2 when used for delivery of shRNA for targeting mineralo-corticoid have shown of receptors for 3 weeks of infection and prevented renal function loss (Rana et al., 2013). Vectors containing lentiviral have been used for delivery of gene in to enhance GFP had induced transgenic expression in the corticomedullar and cortical regions of kidney (Khan et al., 2013). For renal transplantation lentivirus significantly induced slicing of gene but may lead construction of unwanted vital gene region (Ahmad et al., 2008).

Remedial applications of silencing ribonucleic acids

Injury to renal tissues are mostly due to reactive free oxygen production or apoptosis, so proteins stress mediators are potential targets for the delivery of silencing RNA. Nf-kB is a pro-inflammatory factor for the immune cells infiltration of kidney during the acute kidney injury and secretions of pro inflammatory cytokines (Akash et al., 2011). Intravenous administration of specific silencing RNA reduces renal dysfunction significantly as well renal ischemia and nephrotoxicity (Akhtar et al., 2010). Cytotoxic T cells (CTL) infiltration into donor after renal transplantation can result in apoptosis induction as was reported in injection of silencing RNA in local renal vein in animal models in inhibition of C3 by silencing RNA alone or in combination of both to reduce injury of renal reperfusion and protection against renal ischemia (Asad et al., 2011).

Tubulo-interstitial compartment fibrosis is most chronic kidney diseases complication i.e. diabetic nephropathy and glomerulosclerosis, allograft dysfunction contributor (Asad et al., 2012a). Central player of the tubule-interstitial fibrosis is the Transforming growth factors but shRNA reduced it sufficiently (Asad et al., 2012b). Silencing RNA has significantly reduced fibrosis (Farzana et al., 2011a) and glomerular matrix deposition (Chen et al., 2008) in mice susceptible to renal disease due to autoantibody production and activation of complements (Farzana et al., 2011b; Farzana et al., 2011c) lead to glomerulosclerosis.

Analysis of screen image for cellular response has been used as key for interaction of signaling pathways i.e. using protein kinase and silencing RNA knockdown for cells adhesion complex and morphology of cells and proliferation regulation of cells (Farzana et al., 2011b). Sample handling, data management and analysis, imaging equipments require careful attention and consideration for screening analysis (Khalid et al., 2011a).

In vitro models based on one or more pathological conditions may be used as source for studies i.e. nephrotoxicity by determination of caspase activities and binding of annexin-V (Khalid et al., 2011b; Khan et al., 2012; Nasim et al., 2013; Razi et al., 2011; Rehman et al., 2012). Proteins involvement in the induction of fibrogenesis can be determined from actin of smooth muscles and fluorescence intensity for quantitatively measurement of changes in expression (Shabbir et al., 2012).

For the development of cells and cells-matrix interaction, functional characteristics and differentiation a three dimensional environment is required for stimulation (Sher et al., 2012). These 3D models are applicable to RNAi screening of shRN and silencing RNA in immunolabelling analysis.

Conclusion

Targeting of kidney is comparatively easy to that of muscles, brain or tumor cells for uptake of silencing RNA. PEGylation seems not to be more beneficial for delivery of tubular targeting but low immunogenicity and resistance to degradation by nucleases make it more beneficial for treatment of renal injury. Studies indicated that glumerulus and epithelium can be targeted with silencing RNA due to modification in its structure that is helpful in silencing RNA uptake. The major silencing RNA advantage is its target specificity and blocking of down-streaming signaling pathways. Screening of shRNA and silencing RNA libraries in cell models will be helpful in identification of new candidates and their *in vivo* functions.

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