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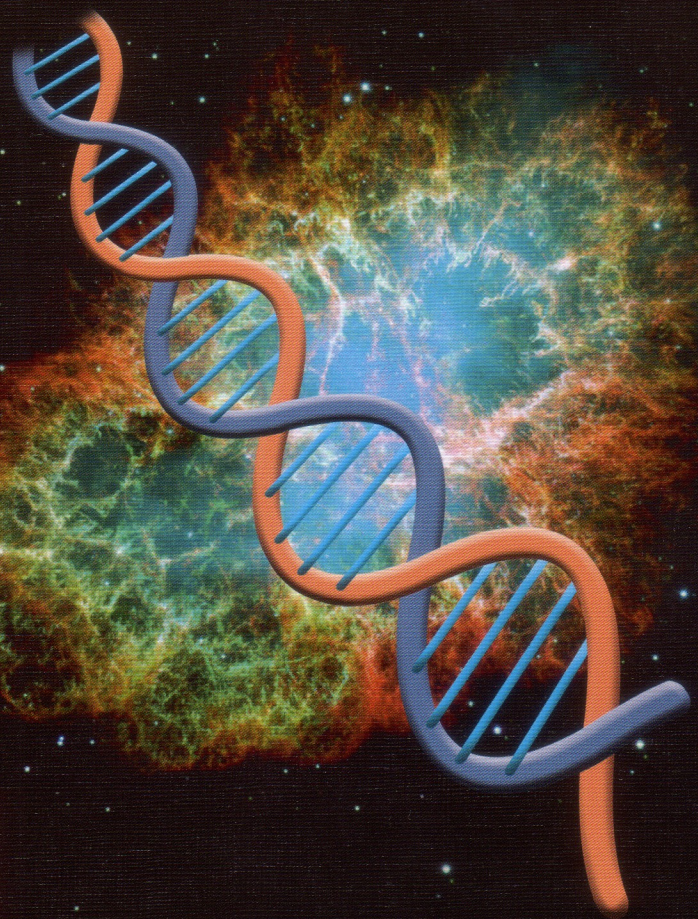

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RNA Interference in Research and Therapy of Renal Diseases

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Abstract

Significant improvements have been made during the last 20 years in therapy of renal diseases including the broadening of treatment options. Gene therapy is a potential modality for many renal diseases for which we are yet unable to offer specific treatment. Here, we introduce RNA interference (RNAi), one type of posttranscriptional gene silencing, as a novel gene therapeutic possibility and describe the mechanism and kinetics of action. We highlight the correlation between structure and efficacy of small interfering and short hairpin RNAs that are the most often used small RNAs possessing RNAi activity. Delivery is the biggest obstacle for RNAi-based gene therapy. Although hydrodynamic treatment is effective in animals, it cannot be used in human therapy. Possibilities to achieve site-specific and effective delivery are listed. Side effects of RNAi and potential solutions are also summarized. Besides the above-described world of small RNAs, we draw attention to the yet unrevealed function of human microRNAs that are localized mainly in the noncoding regions of the genome, are highly conserved among animals and possess important regulatory functions. Although there are many unanswered questions and problems to face in this new field of gene therapy, we summarize a number of experiments targeting renal diseases with the aid of RNAi. High specificity of short interfering RNAs and short hairpin RNAs raise hope for treating renal diseases.

RNA interference (RNAi) refers to the function of double-stranded RNAs (dsRNAs) to cause sequence-specific degradation of complementary messenger RNA molecules leading to selective inhibition of protein synthesis. Being a highly conserved mechanism, RNAi is a common tool among plants and animals to regulate gene expression. One of the most exciting and developing field of RNAi is the harnessing of short interfering RNAs (siRNAs) to develop new therapeutics for human diseases. The potential of RNAi in research and

therapeutics has been honored by the awarding of the 2006 Nobel Prize in Medicine to Craig Mello and Andrew Fire for their contributions to the discovery of RNAi. Until 2007, seven human clinical trials have been initiated utilizing siRNAs [1].

In the postgenomic era, it has been increasingly recognized that a substantial portion of the human genome is not coding proteins. A group of noncoding RNAs, such as microRNAs (miRNAs), is recognized to play an important role in gene expression regulation. Sites and regulatory targets of most human miRNAs remain to be identified.

History

In 1928, it was noticed that tobacco plants infected with tobacco ringspot virus were resistant to the virus in the upper leaves and more than six decades later, RNAi was first described in petunia [2] as a form of protection of the genome from viruses and transposable elements. Externally administered molecules were capable of changing the expression of host's genes ('cosuppression'). In 1998, RNAi was described in the worm *Caenorhabditis elegans*. Grishok and Mello [3] used antisense RNAs in *C. elegans*, the first animal model of gene silencing, showing that introducing long dsRNA into *C. elegans* led to the targeted degradation of homologous mRNA. Later, the same mechanism was described in insects. Administering the sense and antisense RNA strands of dsRNA together led to ten times more effective silencing than using one strand alone. This process was termed posttranscriptional gene silencing and was thought to be related to cosuppression. After recognition of RNAi in lower eukaryotes, attention of biomedical research has been drawn to RNAi by the discovery of its occurrence in mammalian cells. Elbashir et al. [4] showed that RNAi could be induced by siRNAs in mammalian cultured cells. Until today, siRNAs have already been successfully used for gene silencing in numerous animal models, such as nematodes, Drosophila, zebrafish, mouse and rat, but its physiologic role in mammalian species is still not completely understood.

Mechanism

Mechanisms that silence unwanted gene expression are essential for normal cell function. dsRNAs are often produced during the life cycle of viruses, which are eliminated via RNAi. Thus, the in vivo function of RNAi is the host's protection from viruses and foreign genes. Although RNAi is an ancient

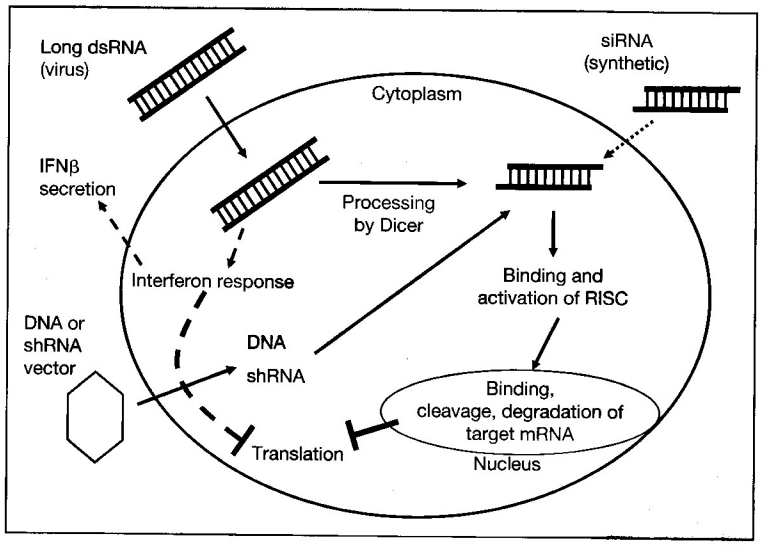

Fig. 1. Summarized scheme of RNAi-cell interaction (black arrows: physiologic pathways, dotted arrow: artificially induced RNAi, dashed arrows: RNAi side effects).

antiviral defense in plants, its role in the natural antiviral defense of mammalian cells is not yet clear. Besides gene silencing, RNAi might be involved in other phenomena of gene regulation. It appears that RNAi is also involved in cell death, development, and gene regulation through DNA methylation.

Long dsRNAs are processed into siRNAs (19 bp of paired RNA with two nucleotide overhang at the 3′ end) by an enzyme called Dicer (an Rnase III ribonucleases). SiRNAs are recognized and incorporated into a multisubunit ribonucleoprotein complex called RNA-induced silencing complex (RISC) with helicase, exonuclease, endonuclease, and homology-searching domains. The RISC is activated upon binding siRNA; thus siRNA duplex is unwound by the helicase, and the sense strand is lost, while the antisense strand directs target mRNA recognition and cleavage [5]. Finally, the endonuclease cleaves the target mRNA. Due to the cleavage of mRNAs, the whole process of transcription and translation is interrupted. In other words, protein synthesis is inhibited without any effect on the genome.

The four consecutive steps of RNAi are: processing dsRNA into siRNAs, incorporation of siRNA into the inactive RISC, unwinding the siRNA duplex, and recognition and cleavage of the mRNA target (fig. 1).

Specificity and Efficiency

SiRNAs are widely used in functional genomic experiments due to their high level of specificity. Properly designed siRNAs can efficiently target a mutated gene, leaving the unmutated form intact. Mostly, all of these specific siRNAs are chemically synthesized. Specificity of small RNAs begins with the proper selection of target genes and target sequences. Optimally, more than one (3 or 4) siRNA sequences should be chosen for the same target sequence to achieve >90% knockdown of the target protein. By experimental design, positive and negative control siRNAs are essential. The appropriate positive control is targeted against a gene naturally present in the host genome, and its silencing can demonstrate that RNAi actually works in the chosen experimental setting (for example β-actin). Negative control siRNAs have the target sequence with a few (2–3) mismatches.

The structure of siRNAs might have a huge impact on their activity. SiRNA duplexes that contain an overhang on the 3′ antisense strand show improved functionality, while an overhang on the 3′ end of the sense strand leads to reduced silencing. Although a single mismatch in the middle of the siRNA duplex is able to prevent target RNA cleavage, more changes are tolerated in the 3′ end.

Despite the high degree of specificity, nonspecific effects of siRNAs have also been described. These include off-target effects, interferon response, and complete shut-down of the protein translation in the target cell (see later). At present, unwanted effects are largely counteracted by appropriate design and in vitro testing of the sequences.

For research purposes, general guidelines for designing highly specific siRNAs are summarized and reviewed elsewhere. Besides the many software packages that design highly efficient and specific siRNAs to prevent nonspecific effects and to enhance specificity, specialized companies (Dharmacon, Invitrogene) supply ready to use sequences to a large library of targets together with appropriate controls.

Comparison with Oligonucleotides

Oligonucleotides (ONs) are short, synthetic single strand RNAs or DNAs that are complementary to any chosen target sequence. A special group of ONs comprise antisense RNAs which hybridize to target mRNA sequences and specifically block translation by sequence-specific cleavage of the mRNA via RNaseH. Before the era of short RNAs, asONs were used for gene loss of function studies. The great advantage of siRNAs over asONs is that siRNAs are

Table 1. Similarities and differences between ONs and siRNAs

Similarities	Differences
Short nucleic acids	ONs are DNAs or RNAs, while siRNAs are RNAs
Common methods in today's laboratories to evaluate/change gene function	ONs are single-stranded, siRNAs are double-stranded molecules
Properties can be altered by introducing modified bases	ONs need no further intracellular processing from precursors, while siRNAs are 'diced'
Similar biodistribution profiles	Effect of siRNA is mediated by RISC, while ONs act by activation of RNase H or steric inhibition
Similar delivery methods available	
Bind to target RNAs via Watson-Crick hybridization	

much more resistant to ribonucleases in the plasma. AsONs could not be measured after administering in vivo, due to their low resistance to nuclease degradation [6]. With the aid of chemical modifications the degradation of siRNAs can be further reduced. For similarities and differences between ONs and siRNAs, see table 1.

Kinetics of the Silencing Effect

Degradation of mRNA is determined by the transcription rate of the target mRNA. Consequently, protein level of the targeted gene depends on mRNA translation rate and half-life of the protein. The loss-of-function phenotype can be detected only at a threshold of protein level.

The kinetics of siRNA effect is determined by

- The duration of the cell cycle (doubling time): In rapidly dividing cells, dilution of the siRNA due to cell division can be a significant factor. Protein level recovered within less than a week in rapidly dividing cell lines such as cancer cell lines, but it took more than 3 weeks in nondividing fibroblasts [7]. Thus, rapidly dividing cells need multiple treatments.
- siRNA dosing schedule: In nondividing cells, the maximum duration of silencing is approximately 3–4 weeks after 1–3 siRNA transfections.
- siRNA properties: If siRNA half-life is shorter than the cell doubling time, dilution due to cell division will no longer be a dominant factor on the duration of gene silencing.

- Protein half-life: Proteins to be silenced with longer half-lives than siRNAs show a slower initial response to the therapy.
- siRNA delivery method: The influence of delivery on silencing kinetics is detailed in the next chapter.

Regulatory RNAs and RNAi

miRNAs are small (approximately 22–25 nucleotide), noncoding RNAs that play an important role in posttranscriptional gene regulation. In plants, miRNAs perfectly match and consequently degrade mRNAs, while in animals they bind imperfectly to 3′ untranslated regions of mRNAs and attenuate protein synthesis at the translational level. For example, miRNAs control the developmental timing and the transition from larval to adult stages of worms. MiRNA genes are located in the introns of or outside of genes and may constitute over 1% of a genome. MiRNA genes are mostly conserved in related species, and many of them are conserved in distantly related species as well [8].

The primary transcript (pri-miRNA) is processed in the nucleus into a stem-loop structure (pre-miRNA) by an endonuclease. Pre-miRNAs are exported into the cytoplasm, where Dicer cleaves the hairpin structure into a 21- to 25-nucleotide mature miRNA. Mature miRNAs are incorporated into the miRNP ribonucleoprotein complex (similarly to RISC – see above). Actions of miRNAs include the cleavage or the translational repression of target mRNA depending on the degree of complementarity between the miRNA and the mRNA. Up to now, miRNAs have been shown to be involved in cell signaling, cancer, maintaining the pluripotent state of stem cells and development [9].

Delivery

Delivery strategies should be considered from several aspects, since different methods are available for in vivo (animal) or in vitro (cell culture) experiments. In the case of cells, delivery methods used for ONs (electroporation, cell microinjection, lipophilic or viral transfection) can be harnessed. Transport of siRNAs across the cell membrane can be facilitated by linking siRNAs to lipids, proteins or basic peptides. Cell-specific delivery by linking siRNAs to cell surface receptor ligands or antibodies could reduce systemic dose and thus potential toxicity [10]. Even hard to transfect cells (such as primary CD4 T lymphocytes) were shown to be transfected by linking siRNAs to protamine-antibody fusion protein with a favorable half-life [11].

For longer duration of silencing, siRNAs can also be expressed in the target cell. Viral (such as adenoviral, adeno-associated viral, oncoretroviral, lentiviral vectors) and nonviral (liposomes, nanoparticles or peptide-lipid complex) expression systems supplemented with complexing of antibody fragments or tissue/cell specific receptors (for more information see relevant chapters) enable site and/or cell-specific siRNA delivery.

Viral Vectors

Almost all types of viral vectors have already been harnessed for RNAi. Adenovirus (especially type 5 adenovirus), adeno-associated virus, retrovirus and lentivirus vectors are most commonly used viral vectors. Viral vectors have the benefits of wide tissue and cell specificity, the ease of production and use. Host immune response resulting in the production of neutralizing antibodies gives the major disadvantage [12].

Nonviral Delivery Strategies

Therapeutic benefit from in vivo delivery of siRNAs has been demonstrated in mice. Synthetic siRNAs can be delivered in vivo using a modified 'hydrodynamic transfection method' which is a high-pressure injection method originally developed to deliver asONs and plasmid DNA. In rodents, siRNAs are rapidly (within seconds) injected intravenously (tail vein) in large volumes (50–100% of the circulating blood volume of the animal), leading to fluid back-up in the venous system of the vena cava, establishing a venous and capillary pressure in parenchymal organs with high blood flow (liver, kidney, etc.). This way, siRNAs were taken up by ~90% of hepatocytes and silenced Fas mRNA and protein in the liver by ~80–90% [13]. Similar silencing effect has been observed in the kidney in mice [14].

Although effective in mice, hydrodynamic treatment is not applicable in human therapy. Regional delivery of siRNAs in smaller volumes of injection into tissues or catheterization of regional veins may be an alternative. It has already been demonstrated that siRNAs can be delivered into the central nervous system, the subretinal area, and the peritoneal cavity in humans.

Expression Plasmids

Small RNAs can be efficiently produced by plasmid vectors which generate approximately 4×10^5 copies of transcripts per cell. Sense and antisense

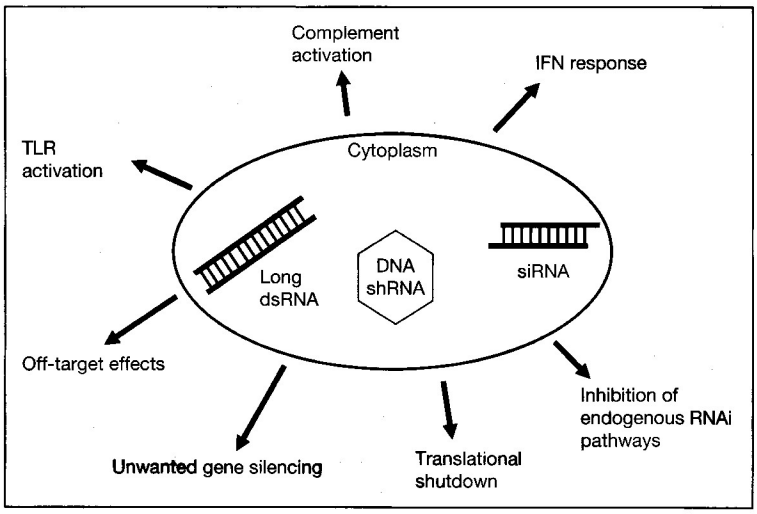

Fig. 2. Possible side effects of RNAi.

strands can be expressed separately (in the case of siRNAs) or in a single transcript separated by a short loop of 5–10 nucleotides that undergoes Dicer processing to become 21-nucleotide siRNAs. MiRNAs can also be expressed by plasmids.

Side Effects, Obstacles (Fig. 2)

Delivery

Delivering Small RNAs into the Targeted Organ/Cell and the Right Intracellular Compartment

Although chemically synthesized siRNAs are cheap, easy to synthesize, bypass Dicer processing and directly enter into the RISC complex, delivery remains a major obstacle for RNAi-based therapy because siRNAs do not cross the mammalian cell membrane unaided and due to rapid renal clearance, the in vivo half-life of siRNAs is extremely short (maximally about 10 min). Increasing lipophilicity of siRNAs allows passive diffusion over the cell membrane, while at the same time enhancing nuclease resistance.

Within the cell, siRNAs should end up in the cytoplasm. Unwanted accumulation of siRNAs within the lysosomes and the nucleus leads to degradation without silencing.

Loss of Effectivity: Escape Mutants

For the antiviral application of RNAi viral mutations may lead to escape mutants. If highly specific siRNAs are used, several siRNAs should be targeting multiple viral sequences.

Off-Target Effects

Unwanted Silencing

Due to perfect base pairing between siRNA and target mRNA, silencing occurs in cells and gene expression is reduced. High (or even low) concentrations of siRNAs may trigger off target silencing: the unintended knockdown of partially complementary sequences. SiRNA design, the use of 3–4 different sequences targeting the same mRNA and careful testing of different sequences in animal models may overcome this problem [15].

Activation of Unwanted Genes

Since almost all kinds of side effects induced by siRNAs have been shown to be concentration dependent [16], the applied amount of siRNA must be always determined in pilot studies. At concentrations of 100 nM, siRNA nonspecifically induced a significant number of genes, many of which are known to be involved in apoptosis and stress response. Reduction of the siRNA concentration to 20 nM eliminated this nonspecific gene activation. Effective siRNA duplexes produce potent silencing at 1–10 nM concentrations [17].

Activation of the Innate Immune System through Toll-Like Receptors

Toll-like receptors (TLRs) play an important role in mammalian innate immunity by recognizing pathogen-associated molecular patterns on the cell surface or within the endosomes, such as bacterial wall endotoxin (LPS), viral dsRNA or cytosine-guanine motifs (CpGs). Several sequences, such as GU dinucleotides or GUCCUUCAA and UGUGU are responsible for activating the innate immune system through TLRs. TLRs also recognize double-stranded siRNAs and consequently TLR intracellular signaling pathways are activated. TLR-3, 7 and 8 have a role in dsRNA-induced signaling.

Cell surface and endosomal TLR3 can be found on myeloid dendritic cells, natural killer cells, neural cells and astrocytes, and responds to dsRNA, a byproduct of viral replication, synthetic siRNA, and poly-inosinic-cytidilic acid /poly(I:C/). Downstream signaling of TLR3 induces the production of type I

IFNs, IL-6, IL-8, IL-12 and TNF-α and induction of sequence-independent gene suppression. This pathway alone is not the only mechanism of innate immunite activation by siRNAs, because siRNA internalization and endosomal maturation is also needed for immune stimulation [18].

TLR7 and 8 are mainly found in the endosomes of antigen-presenting cells and are responsible for recognizing GU-rich single-stranded RNAs, liposome-coated RNAs and siRNAs. Downstream signaling leads to immune activation (IFN-α production) which is absent in siRNA-treated TLR7 knockout mice.

Complement Activation

There are 3 pathways of complement activation: the classical pathway is the antigen-bound antibody-induced activation of the C1 complex, the alternative pathway is initiated by the direct hydrolysis of C3, whereas the lectin pathway is similar to the classical one, but instead of antigen-bound antibody complex, it is initiated by mannose-binding lectin. Activation of C3 is the common step in the 3 pathways.

The alternative pathway is considered to be constitutively active but under normal conditions it is suppressed by negative regulatory components (such as factor H). Factor H is a soluble glycoprotein that circulates in human plasma. Factor H binds to negatively charged glycosaminoglycans. Fluid-phase and surface-bound polyanions (such as small RNAs or other ONs) may mimic the effects of glycosaminoglycans and thus, may deplete factor H, or may detach C3b from factor H resulting in activation of the alternative pathway [19].

The complement system has been shown to be activated selectively through the alternative pathway by intravenous infusion of high-dose phosphorothioate ONs in monkeys [20]. Changing dose and infusion rate revealed that there is a minimum threshold concentration (50 μg/ml) for factor H depletion and consequent complement activation.

Furthermore, oligodeoxyribonucleotides encapsulated in cationic liposomes have also been shown to alter complement activity in monkeys. These changes were attributed to the liposomes rather than ONs [21].

However, intravenous administration of nanoparticles containing siRNAs did not induce complement in nonhuman primates [22].

Interferon Response

DsRNA induces the expression of IFNs directly by activating interferon responsible factor 1, which in turn induces the expression of IFN-α and IFN-β

genes. Interferons are multifunctional cytokines that modulate host immunological functions and inhibit virus multiplication. Most dsRNAs or viral infections induce type I IFNs (IFN-α and IFN-β). IFNs induce IFN-stimulated genes in neighboring cells which contain IFN-stimulated responsive element in their promoter regions. A single molecule of dsRNA (formed during most viral infections) is sufficient to induce IFN synthesis.

Until 2003, it was held that siRNAs are too small to induce interferon response. Sledz et al. [17] discovered that siRNAs designed against different targets activated the interferon response in vitro. Cells infected with lentiviral vectors expressing shRNA sequences also led to the induction of the 2′,5′-oligoadenylate synthetase 1 gene. However, we were not able to demonstrate similar induction of OAS or other IFN-related genes in vivo using small (2 nmol) doses of 2 different 21-nucleotide sequences (targeting the Fas apoptosis receptor and green fluorescent protein/GFP/) in mice. Similarly, others also demonstrated no elevation of IFN-α and IL-6 in vivo after systemic delivery of Fas and caspase-8 siRNAs.

Translational Shutdown

Duplex RNA molecules in the cytoplasm of cells may trigger a profound physiologic reaction. Cytoplasmatic dsRNA activates the dsRNA-activated protein kinase-R (PKR). 500-bp dsRNAs activated PKR and induced nonspecific suppression in Drosophila and nematodes. The binding of dsRNA to PKR leads to PKR autophosphorylation. Upon activation, two pathways are known downstream of PKR:

1. Activation of NF-κB binding sites via NF-κB leading to IFN-β and other cytokine synthesis.
2. Phosphorylation of the α-subunit of the translation elongation initiation factor leading to the arrest of translation (protein synthesis). Consequently, translation is nonselectively shut down.

As part of the antiviral response, dsRNAs longer than 30 nucleotides activate OAS which catalyzes the conversion of ATP into long oligoadenylate chains which activate ribonuclease L. Active ribonuclease L nonspecifically degrades mRNA to initiate apoptosis, a crucial defense mechanisms to overcome viral infections.

Interference with Physiologic Function of Endogenous Regulatory Small RNAs

A large portion of the RNAs transcribed from the human genome, do not code proteins. These RNAs, such as miRNAs play a regulatory role. It can be

hypothesized, that large doses of short RNAs inserted into cells exogenously or by shRNA-coded overexpression may inhibit the function of regulatory RNAs as these short RNA systems use a common enzymatic machinery (for example Dicer or Exportin).

Administration of liposome-encapsulated siRNAs by a single bolus intravenous injection led to dose-dependent and selective RNAi, and both single dose and long-term administration of siRNAs did not inhibit the synthesis and processing of cellular miRNAs. Thus, siRNAs (in appropriate dosage) do not seem to interfere with regulatory functions of endogenous miRNAs [23].

However, saturation of Dicer and RISC by shRNA synthesized siRNA has been demonstrated and led to severe toxicity (weight loss, liver failure, with serum protein and albumin decrease, ascites, widespread subcutaneous edema or death) in mice. This toxicity was shRNA specific and hepatocyte death was due to the oversaturation of the endogenous shRNA processing machinery [24].

On the other hand, Narvaiza et al. [25] found no difference in the accumulation of miRNAs or pre-miRNAs in murine livers after in vivo shRNA transduction.

Solutions

Local Administration

Local injection avoids many of the systemic side effects of intravenous administration, most importantly the rapid elimination. Local catheterization is a popular approach to increase target tissue concentrations of siRNA, even though it is not always feasible because the target tissue cannot be reached and selectivity to nontarget and target cell types may usually not be predicted.

Atelocollagen

Atelocollagen is a highly purified type I collagen (MW = 300 kDa) with low immunogenicity. Atelocollagen complex is applicable for an efficient delivery of siRNA that allows increased cellular uptake, nuclease resistance and prolonged release, and has low toxicity. siRNA/atelocollagen complex becomes solid after in vivo transplantation and remains so for a defined period thus enabling site-specific delivery of siRNAs. This method has a potential clinical relevance.

Using the most effective siRNA concentration might help to overcome the problem of nonspecific silencing as well as translational stop. Decreasing the

siRNA concentration to 1.5 nM did not reduce the specific silencing effect, only a reduction of the siRNA concentration below 0.05 nM vanished the silencing effect, indicating that siRNAs are extraordinarily powerful reagents for gene knockdown.

To achieve long-term gene knockdown short hairpin RNAs (shRNAs) can be used (to treat for instance chronic infections), while siRNAs may be particularly useful in treating acute viral infections.

Chemical modifications of siRNAs are aimed to solve the problems of delivery due to cell membrane impermeability and biodegradation of the siRNA. Intracellular uptake can be facilitated with the use of poly-2′-hydroxyl or cholesterol modifications. 2′deoxy-2′-fluorouridine, 2′-O-methyl and locked nucleotides demonstrate increased resistance against degradation by nucleases, while siRNAs modified with 2′-flouro (2′-F) pyrimidines have a greatly increased stability and a prolonged half-life in human plasma as compared to 2′-OH containing siRNAs. Moreover, 2′-F containing siRNAs are functional in mice and are able to inhibit the expression of a target gene in vivo.

2′-O-methylation has been proven to result in increased persistence of siRNA activity with no toxicity to cells, meanwhile siRNAs with a general 2′-O-methylation in either strand have no activity. Methylation of the sugar moiety or thiolation of the backbone are also well tolerated, causing only a marginal reduction in silencing effect. Toxicity is, however, observed with longer stretches of phosphorothioates, but not with the same level of methyl modification.

Abrogation of the TLR activation is also possible by using chemically modified siRNAs. 2′-fluoro-pyrimidine-modified, nuclease-resistant siRNAs did not activate lymphocytes. Also locked nucleic acids incorporated into siRNAs decreased immune activation. Immune recognition of siRNAs through TLRs can also be abrogated by replacing 2′-hydroxyl uridines with either 2′-fluoro or 2′-deoxy uridines.

Cationic delivery systems, such as polyethylenimines (PEI) are synthetic, cationic polymers that bind and condense the ONs into complexes which are effectively taken up by endocytosis. Recently, PEI-siRNA complexing led to increased resistance of siRNAs against enzymatic and nonenzymatic degradation both in vitro and in vivo. Even though PEI transfection is transient, PEI/siRNA effects were stable for at least 7 days.

Nanoparticle-sized polyplexes modified with arginine-glycine-aspargin ligands (nanoplexes) offer tissue-targeted siRNA delivery into the cytoplasm. Intravenous administration of nanoplexes (containing siRNAs targeting neovasculature integrin expression) into tumor-bearing animals showed sequence-specific inhibition of the target gene and consequential reduction in angiogenesis and inhibition of tumor growth [10].

Uncountable types of (cationic) liposomes and other delivery reagents are becoming increasingly popular both in vitro and in vivo and are commercially available (such as Lipofectamine, Oligofectamine, TransGene, RNAifect, siPort Lipid, monocationic lipid 1,2-DiOleoyl-3-trimethylammonium-propane/DOTAP/, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride/ DOTMA/) that provide high level of transfection efficiency and transgene expression in a wide range of cell types [26].

Even though the frequently used liposomes are an easy and reliable method for siRNA delivery, intravenous administration of cationic liposomes/ siRNAs has been shown to induce IFN response and activation of STAT1.

MPG is a cell-penetrating peptide that can bind any negatively charged molecule via ionic interactions in a nonspecific manner; consequently, it is compatible with almost any given single- or double-stranded ON. MPG is capable of specifically translocating siRNA into mammalian cells by endocytotic processes. Temperature might also have an important role in siRNA/MPG-complexed delivery, since lowering temperature reduces flexibility and fluidity of the plasma membrane, thereby slowing membrane traffic and blocking endocytotic processes.

Therapeutic Applications

Similarly to antibiotics, which target molecules essential for prokaryotic development, but not involved in the eukaryotic system, the optimal targets for siRNA mediated gene-therapy are well characterized targets, foreign to the human body. Furthermore, systemic side effects can be minimized by targeting organs sequestered from the blood circulation. Thus, at present siRNA-based gene therapy is closest to clinical application in viral infections, malignant and ocular diseases. Besides, siRNAs have already been tried therapeutically in a broad range of diseases both in vitro and in vivo, such as bacterial and viral infections, autoimmune diseases, hypercholesterinemia, neuropathic pain, neurodegenerative diseases, cancer, septic shock and even sexually transmitted diseases [for more information, see 27]. Transplantation however, may also emerge as a potential field, due to the ex vivo phase of the graft, which provides an ideal window for the organ-specific knockdown of certain pathology-related proteins, without the necessity of introducing the short RNA into the systemic circulation. Thus, more effective transduction protocols may be applied than in vivo, and by washing out the graft before implantation, the vehicle as well as the siRNA which is not taken up by the cells can be removed, eliminating potential systemic side effects.

Apoptosis Regulation in Renal Ischemia/Reperfusion Injury

It is well-known that ischemia/reperfusion injury (IRI) is central to many pathophysiological conditions such as transplantation or acute renal failure and is a leading cause of death of patients in sepsis/shock. Cell death during ischemia is predominantly necrotic, whereas during reperfusion, apoptotic. The extension of cell death during reperfusion may be larger than during ischemia.

We investigated the effect of silencing Fas expression within the kidney of mice and observed that mice receiving Fas siRNA had better survival and renal function than those receiving saline or indifferent siRNA. Thus, local and systemic injection of Fas siRNAs (even if administered after ischemia) protected mice from ischemia-reperfusion injury. Similarly, complement-3 and caspase-3 expression was markedly diminished by specific siRNA treatment which protected against lethal IRI by preserving renal function in mice. Furthermore, viral delivery of shRNAs targeting Fas and caspase-8 also protected mice from IRI, indicating a therapeutic potential of genetic knockdown of proapoptotic proteins in kidney donors by RNAi in transplantation [28]. Finally, RNAi targeting Fas [13] or caspase-8 [29] protected mice from fulminant hepatitis as well as from sepsis in the cecal ligation-puncture model as demonstrated by reduced apoptosis in liver and spleen, lower plasma liver enzymes and a survival benefit.

Chronic Kidney Diseases

Mesangial cell hypertrophy was inhibited in an experimental model of diabetic nephropathy, by RNAi inhibiting p8: an endothelin-induced molecule [30]. An increasing number of mutations known to be responsible for both inherited and sporadic forms of polycystic kidney disease – the most important inherited cause of end-stage renal disease may represent potential targets for RNAi-based therapy. Transient knockdown of Smad proteins using RNAi resulted in complete inhibition of TGFβ1-induced tubulointerstital fibrosis.

RNAi and Renal Tumors

Von Hippel-Lindau (VHL) disease is caused by the inactivation of the VHL tumor suppressor gene leading to multiple hemangioblastomas and clear-cell carcinoma of the kidney. The VHL gene product inhibits hypoxia-inducible factor 2 (HIF2α). Under hypoxic conditions, HIF2α induces vascular endothelial growth factor, platelet-derived growth factor B, transformation growth factor-α, epidermal growth factor or matrix metalloproteinases. Inhibition of

HIF2α by shRNAs expressed by a retrovirus vector sufficiently inhibited tumor formation induced by VHL gene product-defective renal carcinoma cells in mice.

Prognosis of renal cell carcinomas (RCCs) is poor due to their high metastatic ability. IFN-α2 has been shown to increase the survival of patients with metastatic RCCs due to its apoptosis-inducing effects. Clinical antitumor effects of IFNs may be augmented in RCC and melanoma by targeting DNA methyltransferase 1 with RNAi technology which results in the reactivation of methylated and thus, inactive tumor suppressor genes (such as Ras association domain family 1A gene).

Resistance to Chemotherapy

Tumors that fail to apoptose after DNA damage escape death after exposure to chemotherapeutic drugs leading to the failure of chemotherapy. Knockdown of phosphatase and tensin homolog detected on chromosome ten (PTEN) results in increased stability and cytosolic localization of the cell cycle protein p21 which is associated with the regulation of cell death and regeneration after DNA damage; thus, p21 is thought to be responsible for resistance to apoptosis. Chemotherapy resistance may be reduced by siRNAs targeting PTEN in combination with chemotherapeutic agents to overcome chemotherapy resistance.

Small monomeric GTPases of the Ras superfamily play an important role in the control of excessive proliferation of cells, apoptosis, migration, adhesion, contraction, secretion, and receptor expression in renal diseases. Targeting Ras genes in renal therapies might serve as a therapeutic tool. Renal cell proliferation might be sensitive to downregulation of Harvey Ras and Kirsten Ras by the use of RNA-interacting agents such as asDNA and siRNA. Ras targeting has reached the clinic in a phase 2 clinical study of treatment of pancreatic cancer; consequently, it may be a useful therapeutic alternative also for renal diseases.

Conclusions

RNAi-mediated gene therapy has already reached the clinic. Safety and efficacy of siRNAs are being assessed via clinical trials in age-related macular degeneration, preeclampsia and chronic myeloid leukemia [1]. On the other hand, there are still several obstacles to overcome, such as proper delivery or possible side effects. Special delivery methods and chemical modifications of siRNAs offer help for researchers to solve the above-mentioned problems to enable RNAi to become a common therapeutic option for clinicians in treating infectious, inherited or malignant diseases.

Acknowledgements

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Initially conceived as a strategy to remedy inherited genetic disorders, gene therapy has been successfully applied in the last decade to ameliorate the renal function compromised by progressive renal diseases and to prevent kidney allograft rejection in experimental animals.

In the present book, world-renowned experts are presenting new insights into viral and non-viral systems used to effect gene delivery, one chapter being dedicated to the new field of RNA interference (RNAi). This latter method may be successfully used in renal ischemia-reperfusion injury, trauma and transplantation. In the near future, gene therapy might also prove to be a new strategy to target molecules involved in tissue damage and inflammation processes that underlie ARF. So far, gene transfer has been successfully applied in experimental glomerulonephritis and interstitial fibrosis, and studies in larger animals are keenly awaited. Also covered are issues related to transplantation, which is the therapy of choice in many end-stage organ failures. Transfer of genes whose protein products have immunomodulatory properties have proven beneficial in treating acute and chronic graft rejection, one of the problems not satisfactorily solved by current anti-rejection drugs. Gene therapy thus may become a reality in clinical transplantation once its efficacy in larger animals has been demonstrated. Last but not least, a possible benefit of targeted gene therapy in renal cancer or HIV-associated nephropathy is explored.

Covering a wide spectrum of topics, this publication provides a valuable overview of current developments and issues.

Cover illustration: In 1054, Chinese astronomers registered the appearance in the sky of a 'new star' which remained visible for 21 months and then disappeared. The recent identification of the Crab Nebula in the Taurus Constellation sheds some light on the enigmatic disappearance of the supernova. Experiments in gene therapy date back over six decades to Oswald Avery's pioneering studies with pneumococcal transformation showing that some bacteria can take up naked DNA. Now it is perhaps time to see the impact of correcting genetic abnormalities by means of a gene as treatment for many disabling or devastating diseases.
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KARGER