Pharmaceutical Research, Vol. 21, No. 1, January 2004 (© 2004)

Review

Pharmaceutical Prospects for RNA Interference

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Received August 8, 2003; accepted September 10, 2003

RNA interference has proven to be a powerful tool in gene function validation. Recently, the first studies were published reporting a disease-modulating activity of the technique, suggesting a promise for RNA interference as a novel therapeutic strategy. This review discusses the recent advancements in realizing the clinical utility of RNA-interference.

KEY WORDS: siRNA; RNA interference; drug delivery; nucleic acids; gene silencing.

INTRODUCTION

RNA interference (RNAi) has generated remarkable excitement lately in the biomedical research community (1). Over a decade ago, this gene-silencing phenomenon was first described to occur in plants and fungi (2). In 1998, Fire and colleagues demonstrated that RNAi could also be invoked in animals (3). Its physiologic function appears to lie in viral defense, transposon silencing, and regulation of developmental pathways (4). In experimental settings, RNAi was readily appreciated as a powerful tool in gene function validation experiments (5). In addition to its use in functional genomics, RNAi holds a promise as a novel therapeutic strategy. Its attractive properties include a strong knockdown of the targeted gene for a relatively prolonged period of time and a presumed high sequence specificity. Single nucleotide difference between targets can result in dramatic differences in the RNAi-mediated gene silencing, which is an interesting quality for treatment of several dominant-negative disorders by targeting specific alleles with single nucleotide mutations compared to the wild type (6).

MECHANISM OF RNAi

The mechanism of RNAi is not completely understood. However, the generally proposed model is that a double-stranded (ds) RNA, which is targeted against a homologous mRNA, binds to the mRNA and mediates its degradation, causing the silencing of the corresponding gene. The process has recently been reviewed (6) and is described in Fig. 1.

Between intracellular introduction of the dsRNA and the silencing of the gene, several steps occur inside the cell. The dsRNA is cleaved into smaller duplexes by an RNase III-like activity involving an endogenous enzyme named Dicer. These shorter fragments consist of 19–21 nucleotide duplexes with two nucleotide overhangs on the 3′ position and are known as small interfering RNAs (siRNAs). Subsequently, siRNAs associate to several proteins that together form a nuclease complex known as the RNA-induced silencing complex (RISC). As the siRNA unwinds, probably through the action of a helicase, the RISC becomes activated, and the antisense siRNA strand "guides" the RISC to the complementary target mRNA through Watson–Crick base pairing. Finally, the mRNA is cleaved approximately 12 nucleotides from the 3′ terminus of the complementary siRNA strand, thereby silencing the corresponding gene.

Gene downregulation by RNAi has initially been studied in *Caenorhabditis elegans, Drosophila melanogaster*, and other nonvertebrates. RNAi-mediated gene knockdown in mammalian cells has only very recently been demonstrated (7). The difficulty in showing a sequence-specific RNAi-mediated gene silencing in most mammalian cell types lies in a nonspecific protein production shutdown, which is partly mediated by the RNA-dependent protein kinase pathway, by introduction of long (>30 nucleotides) dsRNA molecules. Only by introducing the smaller siRNA can a sequence-specific gene silencing effect be observed (7). Other important differences between RNAi in mammalian cells and RNAi in plants and *C. elegans* are the occurrence of an amplification step in the nonvertebrates, which is followed by spreading of the silencing effect (6). Presumably, the signal is amplified through the action of an RNA-directed RNA polymerase, resulting in the generation of secondary siRNA and followed by a spreading of the silencing signal throughout the organism. In *C. elegans* it has been demonstrated that the silencing effect can be observed in the first-generation offspring. Mammalian cells apparently lack the machinery that is required for both processes, making RNAi in mammalian cells both transient and confined to the cells in which the siRNA is introduced.

siRNA

siRNA can be introduced in two forms into mammalian cells: 1) via transfection from the extracellular compartment as a chemically or enzymatically synthesized product or 2) as a product of intracellularly transcribed DNA upon transfec-

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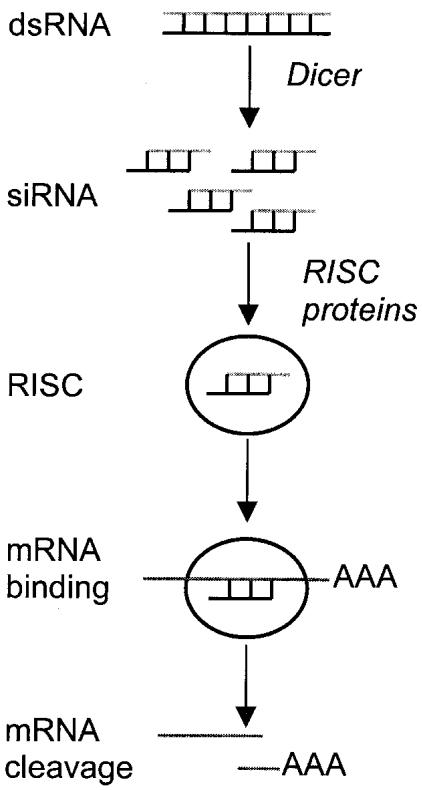

Fig. 1. Proposed mechanism for RNAi. Long dsRNA is enzymatically cleaved by the endogenous enzyme Dicer into fragments of 19- to 21-base pair duplexes with two nucleotide overhangs. Resulting small interfering RNA (siRNA) interacts with several proteins to form an RNA-induced silencing complex (RISC). When the siRNA unwinds and the RISC becomes activated, the siRNA associates to the target mRNA through complementary base pairing. The mRNA is subsequently cleaved. (Nearly) exact base pairing is essential for successful sequence-specific silencing of the homologous gene.

tion, transduction, or infection with the transcript-encoding genetic material.

Tuschl and colleagues have identified many of the requirements and preferred characteristics of siRNA with regard to silencing efficacy (7,8). siRNAs preferably consist of 19 nucleotide duplexes with 2 nucleotide overhangs on each 3′ position. The overhang sequence does not appear to be critical and can even include 2′-deoxynucleotides (like TT), which are thought to be more resistant to the action of nucleases (7,8). However, replacement of either strand with a strand composed of 2′-deoxynucleotides annihilates RNAi activity. Several studies addressed the influence of siRNA nucleotide modifications, which can be introduced to label the siRNA or enhance its stability against degradation. In general, modifications to the 3′ position are better tolerated than modifications to the 5′ positions with regard to preserving silencing efficacy (9,10). The phosphorylation of the 5′end of the siRNA, needed for activity, may explain this difference. Although base modifications may offer the prospect of increased siRNA stability, modified bases may distort normal cell metabolism, requiring careful investigation of possible toxicity.

Because of the high target sequence specificity of RNAi, mismatches between mRNA and the siRNA duplex have been shown to strongly reduce activity (7–11). Mismatches in the center of the duplex, generally, have stronger effects than mismatches closer to the ends of the strand. In addition, mismatches in the mRNA-binding antisense strand of the siRNA duplex generally have stronger effects than mismatches in the sense strand (10). Different regions of the targeted gene can display a different tolerance to mutations in the corresponding siRNA (9). This is probably the explanation for the differences found in effects of mismatch introduction on siRNA activity. For some siRNA, single nucleotide mismatches have been reported to abolish activity, whereas others have reported an absence of RNAi effects only after introduction of 7 mutations (12,13). When, instead of focusing on the target gene alone, all genes are examined for siRNA effects, the picture becomes less clear (14–16). Three conflicting reports have been published thus far: two describing the absence of any interference of siRNA on non-target mRNA (14,15), whereas the other report claims cross-reaction of siRNA with approximately 0.1% of the investigated 21,000 transcripts with even only limited (seven nucleotides) or no sequence similarity (16). The differences between these studies could be the result of siRNA concentration as higher concentrations have been associated with a different pharmacological profile (15). Yet, Jackson *et al.* also reported off-target regulation at very low siRNA concentrations albeit that these effects were far less pronounced than for the higher concentrations (16). Also, the fastest, strongest and most prolonged effects were only seen for the siRNA specific for the targeted gene (16). Another difference between the studies may be the kinetics of on and off rate of siRNAs with specific and nonspecific mRNA-sequences. Clearly, more studies are needed to address this issue. However, in general, it is difficult to imagine a use for a highly conserved biological defense mechanism that would have strong nonspecific effects. Nevertheless, as with all interactions of chemicals with biological systems, absolute specificity is a biological impossibility and some nonspecific effects will occur.

The siRNA synthetic chemistry has rapidly evolved. Current yields of each nucleoside-addition step are >99%, resulting in efficient synthesis of full-length siRNA (17). Sample purity is a key element in obtaining unbiased results. On an experimental scale, most companies can guarantee a sufficient purity grade. Although direct comparisons are lacking, siRNAs synthesized by different methods appear to have similar gene silencing efficiencies. Use of synthesized siRNA has the advantage of offering precise control over the actual amount and purity of siRNA used in the experiment, the possibility to characterize the siRNA, and the ability to introduce modifications into the siRNA to label it or to enhance its efficacy. Intracellular enzymatic production of siRNA or small hairpin RNA (shRNA) via plasmid DNA has the important advantage that cellular exposure of cells can occur for a prolonged period of time, this approach has been recently reviewed (18). For enzymatic production of siRNA via plasmid transfection, the same structural requirements apply as for synthetic siRNA. The approach described in literature uses U6 promoter regions that express both the sense and antisense RNA strand. Supposedly, both strands anneal intracellularly to yield the functional siRNA duplex.

Intracellularly produced shRNA appears to be a more efficient mediator of RNAi than intracellularly produced siRNA (19). The difference is probably related to the need for the complementary siRNA strands to associate into a du-

plex to become active, whereas the complementary regions are connected in shRNA. The shRNA, produced by plasmids containing the RNA polymerase III dependent promoter (H1-RNA) region, consist of 19 nucleotide inverted repeats (forming the hairpin-stem) and a, not strictly regulated, number of spacer nucleotides (forming the loop) (19–21). The 3′ overhang is generally composed of 4 uridine-nucleotides. By cotransfecting such a vector with a gene conferring puromycin-resistance, stable expression can be achieved, which has been reported to last up to two months (22). Similar constructs have been created based on viral vectors for the gene transfer for shRNA production (23).

siRNA SEQUENCE-DESIGN

The most important requirement in the design of siRNA is the alignment of the siRNA sequence to the sequence of the mRNA of interest. Generally, the target is chosen downstream of the start codon in the translated region of the transcript. siRNAs that are chemically synthesized, preferably have the general motif AA(N19)TT (N = A, C, G, U) and a G/C content of approximately 50% (9,10,24). When the sequence is based on this motif, both strands of the siRNA duplex can be synthesized with symmetric 3′ TT overhangs. The symmetry is expected to result in equal ratios of sense and antisense strands interacting with the RNAi-machinery (10). In addition, the antisense strand will be fully complementary to the target sequence. Nevertheless, the motif NA(N21) with a G/C content between 30% and 70% can also yield functional siRNA, as the utmost 3′ nucleotide of the antisense siRNA strand does not seem to play an important role in target recognition and the 3′ overhang of the sense strand does not contribute to specificity.

For enzymatic RNA polymerase III-mediated synthesis the motif NAR(N17)YNN (R = A or G, Y = C or U) is preferred, as the enzyme acts more efficiently when the first transcribed nucleotide is a purine. For both synthesis routes, target sequences should preferably not contain more than three G or C nucleotides in a row as polyG or polyC motifs may hyperstack, which may interfere with correct processing.

Checking the proposed siRNA sequence is critical to avoid chance complementarities to a splicing junction, a single nucleotide polymorphism-region, an unrelated mRNA or a regulatory region. Generally, BLAST sequence analysis programs (http://www.ncbi.nlm.nih.gov/BLAST/) are used for validation of proper siRNA targeting regions against expressed sequence tag (EST)-databases of the desired species.

Despite these rules, pronounced differences exist in the efficiency with which different siRNAs targeted to the same mRNA are able to knockdown gene expression. One of the factors determining the efficacy is the position of the transcribed region that is targeted. Holen *et al.* showed that a shift of only three nucleotides in the position of a target region of the human tissue factor gene can have pronounced effects on siRNA silencing efficacy. The pattern of siRNA-mediated silencing of human tissue factor, as a function of the position of the target region was independent of the cell type in which it was studied (11).

More elaborate analysis of the positioning effect of siRNA on silencing efficacy has been performed within Dharmacon Inc. (Lafayette, CO, USA) for an entire gene as is shown in Fig. 2. Random synthesis of siRNAs that spanned the entire translated region of a gene showed pronounced differences in gene-specific mRNA knockdown at 24 h after transfection. It is speculated that this difference in efficacy is related to the accessibility of the mRNA because of secondary structure formation or because of a sequence-dependent difference in siRNA activity.

For this specific gene, 28% of the siRNAs inhibited mRNA levels over 95% over a 24-h period. Based on analysis of the information for this and several more genes, Dharmacon (Lafayette, CO, USA) has developed an algorithm (SMARTselection™) based on 34 criteria affecting gene-silencing efficacy. Using this algorithm, 97% of the synthesized siRNAs show >50% mRNA knockdown, with nearly half of these inhibiting mRNA levels for more than 95%. By pooling siRNAs selected using this system, efficacy can be further enhanced reaching >99% of the synthesized siRNAs, inhibiting mRNA levels >50% and 85% knocking down mRNA levels for more than 95%. (R. Duong, personal communications). It is important to realize that reduction in targeted protein levels may only become apparent after several days, depending on the protein copy number and half-life. Many studies have demonstrated that non-sequence specific effects can be invoked by some oligonucleotides. Therefore, inclusion of negative controls seems pivotal to confirm the specificity of the observed effects, as has been reviewed recently for antisense (25). It is expected that, in general, scrambled nucleotide sequences of the siRNA, validated to lack significant homology to any expressed sequence tag of the desired species, are valuable controls. A study with a crossover design can also be a relatively simple way to ascertain specificity of two targeting sequences.

IN VITRO USE OF siRNA

In vitro uses simplify many of the problems associated with target cell delivery, target selectivity, homeostasis mechanisms and possible non-target cell toxicity encountered *in vivo*. Yet, the cellular membrane is still an important physical barrier to the efficient intracellular introduction of nucleic acids. Similarly as in gene and antisense studies, passage through the cell membrane of nucleic acids is difficult to achieve as a result of their hydrophilic and polyanionic nature. Experience with DNA and antisense oligonucleotides has provided a variety of chemical and physical techniques that would allow the transfection or infection of cells with extracellularly synthesized siRNA, as reviewed recently (26–28). The different techniques currently available for intracellular introduction of siRNA are schematically drawn in Fig. 3.

The chemical techniques are generally based on the use of (poly)cations. The cationic compounds condense the siRNA, resulting in the formation of particles. These particles generally bear a net positive charge, as an excess of the cation provides efficient condensation and prevents particle aggregation. The net positive charge of the particle additionally promotes electrostatic interaction with the overall negative charge of the cell membrane. Both by condensing the siRNA and promoting cell membrane interaction, relatively efficient cell transfection can be achieved. A whole range of cations exists that have been shown to promote the intracellular delivery of nucleic acids (26–28). Of these, the cationic lipids, cationic polyamines and combinations of both are the most prominent. The ultimate *in vitro* transfection-efficiency of a

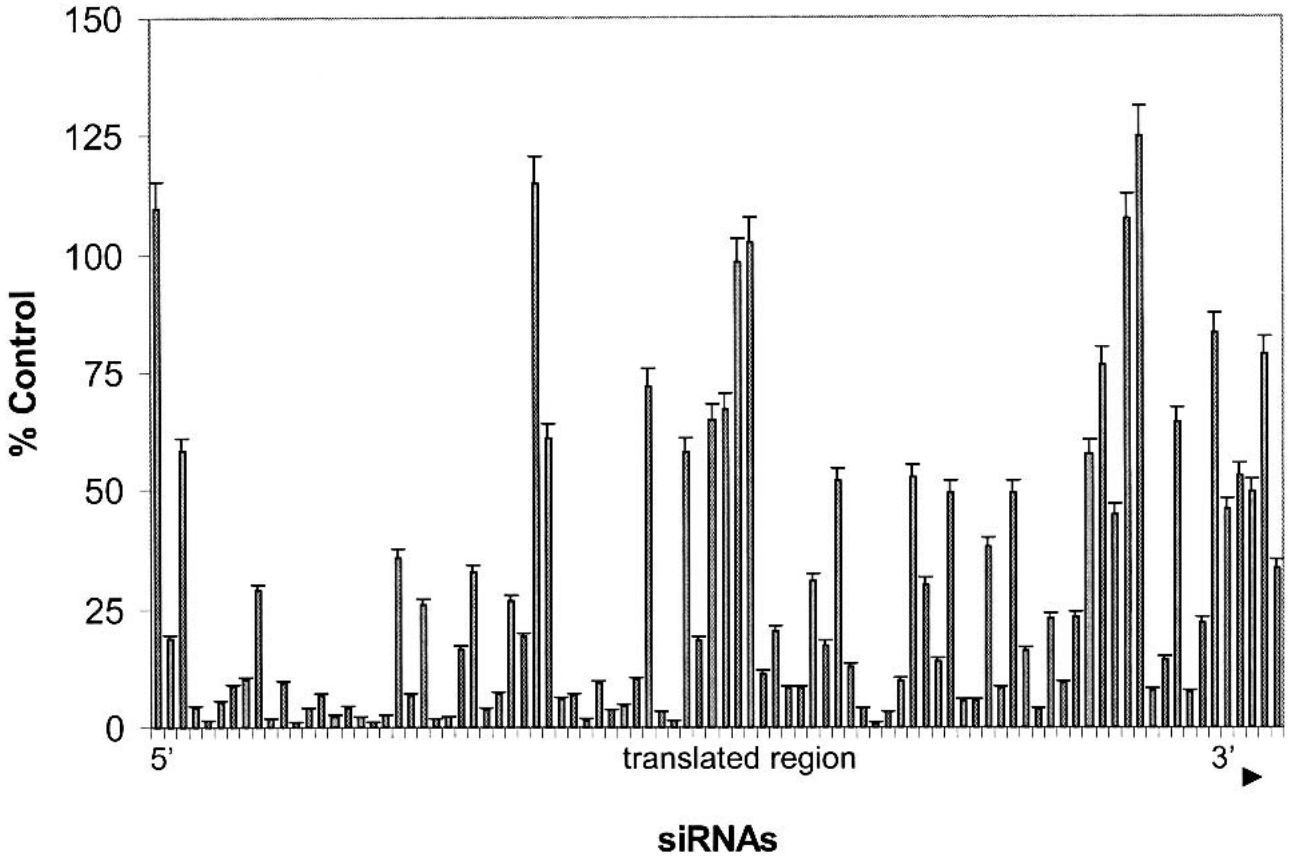

Fig. 2. Investigation of siRNA position dependence for an entire gene. siRNAs, spanning the entire translated region of a gene, were synthesized and each siRNA was transfected using the same method. Gene-specific mRNA levels at 24-h post-transfection are reported as percentage of control values. For this specific gene, 28% of the siRNAs resulted in >95% mRNA knockdown.

cationic compound appears to be dependent on the characteristics of the cell, like cell type, confluency, and passage number, as well as the properties of the cationic particle, such as cell medium compatibility, cell toxicity, strength of electrostatic interaction, cation structure, and particle size. It appears that the most popular cationic agents possess, in addition to their nucleic acid condensing ability, an ability to influence the intracellular processing of the particle.

Although interaction between the cell surface and the delivery system promotes siRNA uptake, appropriate intracellular routing should still follow the interaction. The cationic particles are likely taken up by cells through endocytosis. Therefore, the cationic particle and/or its siRNA should be able to escape the endosomal vesicle to evade lysosomal degradation to allow the RNAi effects to occur. Although, systematic studies evaluating the efficiency of siRNA introduction into cells by various transfection reagents are lacking thus far, it is conceivable that some polycations will have a more suitable structure for the introduction of small siRNA than others.

Physical techniques to overcome the cellular membrane barrier either force the nucleic acid into the cell (like gene gun, magnetofection,) or lower the cell membrane barrier (electroporation, or ultrasound) (28). For transfection of genetic material encoding for transcript siRNAs or shRNAs the same considerations are valid. Recent research suggests that nuclear localization of siRNA produced by transcription may be required for efficient gene silencing (29), although another study reports cytoplasmic degradation of mRNA by externally applied siRNA (30). It has been suggested that the intracellular processing of externally applied siRNA or intracellularly transcribed siRNA may be different (29).

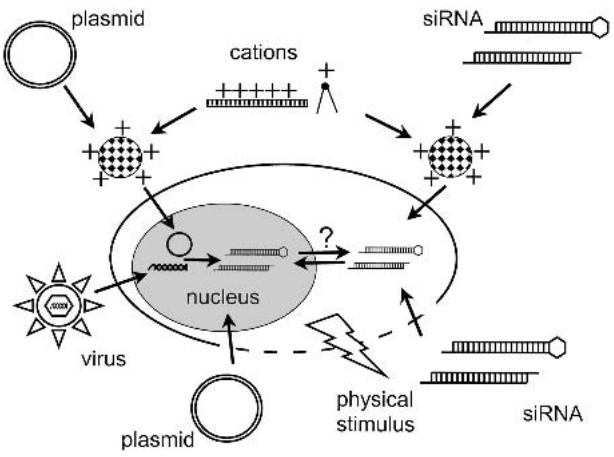

Fig. 3. Introduction of siRNA into mammalian cells. siRNA can be introduced either as a synthetic construct or synthesized by the cell after introduction of the encoding genetic information. Three strategies have been described for nucleic acids to pass the cellular membrane: delivery after complexation to cations, delivery by a virus, or delivery after distortion of membrane integrity by physical stimuli. The appropriate intracellular location of siRNA activity is at present unknown.

For transcript-encoding DNA, additionally, viruses can be used to deliver the genetic material. The viral techniques make use of the evolutionary adaptations of virions for efficient cell attachment and correct intracellular processing for high-level transcription of delivered genetic material. From gene therapy studies useful viruses have been identified including the retrovirus, adenovirus, herpes virus, simbis virus, lentivirus, and baculovirus, each having its own advantages and disadvantages as has been reviewed recently (31). These viral systems can also be attractive for the delivery of transcript-encoding DNA *in vitro*.

Taken together, these systems have provided or could provide means for studying loss-of-function phenotypes, target identification and validation, and identifying possible therapeutic strategies for siRNA *in vitro*. Notable examples of possible therapeutic strategies identified *in vitro* include the knockdown of point-mutated p53 restoring the nononcogenic wild-type phenotype (32) and knockdown of viral infectivity of hepatitis virus, human immunodeficiency virus, and influenza virus (33–35). Still, to assess a protein's importance and potential in disease-control, which is in the ultimate interest of pharmaceutical applications, *in vivo* studies are needed.

IN VIVO siRNA DELIVERY

Whereas soaking of *C. elegans* in a dsRNA solution is already sufficient to observe RNAi effects, for mammals the delivery needs to be more sophisticated. In addition, to the already mentioned cellular barrier, other barriers for *in vivo* effects include target cell selectivity, *in vivo* siRNA stability, homeostasis mechanisms and side effects on nontarget cells. Nevertheless, several reports appeared recently reporting successful *in vivo* use of RNAi in mammals.

An approach that may be of pharmaceutical interest for identification and validation of disease-controlling proteins is the creation of transgenic animals. These animals intrinsically produce specific siRNA in all cells as transfection with the siRNA encoding genetic material usually occurs at the egg or early embryonic states. (36–40). Transgenic eggs of mice and rats, which would normally express green fluorescent protein (GFP) throughout their body, were microinjected with a vector expressing siRNA against GFP. GFP expression was no longer noted in any organ, whereas animals transfected with a vector expressing control siRNA did. F1 progeny also lacked GFP expression (36).

Stein *et al.* used an oocyte-specific Zp3 promoter to express 500-bp long hairpin dsRNA designed against Mos mRNA. Transgenic founder animals displayed the known Mos null phenotype: female infertility (37).

Heritable gene silencing could also be enforced by electroporation of an siRNA expression vector into mouse embryonic stem cells. The vector was directed against Neil1, a protein involved in DNA repair. Implantation of blastocysts/stem cell chimeras and studying of the F1 progeny revealed that approximately half of the animals showed transmission of the siRNA construct (38). A lentiviral approach was also shown to be feasible. Transduction of eggs from GFP-positive transgenic mice with lentivirus-expressing siGFP virus led to reduced fluorescence in blastocysts. More interestingly, F1 progeny, expressing siRNA targeted against GFP, showed reduced fluorescence and GFP levels (39). Rubinson *et al.* also demonstrated that lentivirus-delivered shRNAs created stable and functional silencing of gene expression in transgenic mice (40).

One study directly compared the silencing efficiency of nuclease resistant antisense oligonucleotide and of siRNA against GFP, which was stably expressed in HeLa cells, in cell culture and in xenografts in nude mice (41). Cationic lipid-based Cytofectin GSV was used to deliver both nucleic acids. In cell culture it was shown that the siRNAs were more efficient in silencing GFP and that the siRNA-effect lasted longer than the effects of antisense. In nude mice bearing a HeLa tumor xenograft, 20 μg of siRNA administered intratumorally formulated with Cytofectin GSV reduced GFP expression as determined by GFP antibody-staining on tumor tissue slides. The nuclease resistant antisense oligonucleotides failed to inhibit GFP expression or to reduce mRNA levels. siRNA reduced the mRNA levels to approximately 75% of control values. The absence of efficiency of antisense oligonucleotides is probably due to their lower resistance to nuclease degradation.

Three studies have reported the use of the hydrodynamic pressure assisted delivery of nucleic acids for siRNA. The method consists of rapid administration of the siRNA in an unformulated form in a large volume of physiological saline (approx. 2 ml in mice) (Ref. 42).

Coinjection of 10 μg luciferase-encoding plasmid with 5 μg of synthetic siRNA resulted in 80–90% knockdown of luciferase activity in all organs investigated (liver, kidney, spleen, lung, and pancreas). Interestingly, as little as 0.05 μg of siRNA was able to induce detectable luciferase inhibition. Targeting of siRNA against an enzyme expressed from a long-term expression vector showed that after 4 days approx. 30% inhibition could still be observed, complete return to normal levels was observed after two weeks (43).

Mc Caffrey *et al.* also demonstrated a sequence-specific 70% knockdown of luciferase activity by hydrodynamic co-delivery of 2 μg of plasmid and 40 μg of siRNA. Expression of shRNA directed against luciferase via an RNA polymerase III promoter containing plasmid showed even more pronounced luciferase knockdown (approx. 100%) when delivered at a 10-μg dose together with 2 μg of reporter plasmid (44).

In a study reporting therapeutic efficacy, the Fas receptor was targeted protecting mice from hepatitis complications. Hydrodynamic delivery of 50 μg Fas-targeting siRNA for 3 days specifically reduced Fas mRNA levels and expression of Fas protein in mouse hepatocytes up to 80–90%. These effects persisted for 10 days. Treatment with Fas siRNA 2 days before a concanavalin A-challenge inhibited hepatocyte necrosis and inhibited serum transaminase levels over 90%. After six weekly concanavalin A injections with Fas-targeting siRNA injections at week 2 and week 4, the treated animals were protected from the development of hepatic fibrosis. In a model of liver failure by intraperitoneal injection of agonistic Fas-specific antibody, 82% of mice treated with Fas-specific siRNA survived for 10 days of observation, whereas all control mice died within 3 days. Importantly, siRNA sequences that were directed against Fas but provided little or no silencing efficacy failed to protect mice death (45). In a similar set-up, prophylactically administered siRNA specific for caspase 8 protected mice against the acute liver damage induced by Fas, antibody Jo2 or adenovirus expressing Fas li-

gand. Also in ongoing disease the sequence-specific siRNA proved to be beneficial (46).

Using recombinant adenovirus as the delivery agent for expression of siRNA, specific silencing of β-glucuronidase activity could be demonstrated at a dose of 25 infectious virions per HeLa cell *in vitro*. In the *in vivo* experiments, transgenic mice expressing GFP received 1×10^7 infectious viruses in the brain striatal region effectively silencing GFP expression in the injected hemisphere. Similar results were obtained in the liver for the endogenous β-glucuronidase gene (47).

Laser-induced expression of the proangiogenic vascular endothelial growth factor followed by local injection of cationic lipid complexed siRNA targeted against the mRNA of this protein in the eyes of mice inhibited ocular neovascularization to a quarter of the area involved in angiogenesis in the presence of irrelevant siRNA (48).

Sorensen *et al.* are the first to report a cationic siRNA formulation and systemic application of siRNA *in vivo*. The system is based on the cationic lipid *N*-(1-(2,3-dioleoyloxy)propyl)-*N*, *N*, *N*-trimethylammonium chloride. By codelivery of 50 μg GFP-encoding plasmid together with 30 μg of siRNA targeting GFP in cationic liposomes via intravenous injection, GFP expression in all organs that were transfected could be inhibited as determined by fluorescence microscopy. Unfortunately, the system does not allow control over the target cells. Local intraperitoneal injection of *N*-(1-(2,3-dioleoyloxy)propyl)-*N*, *N*, *N*-trimethylammonium chloride siRNA specific for tumor necrosis factor-alpha inhibited production of this cytokine by peritoneal macrophages, as these cells did not respond to lipopolysaccharide challenge, while stimulation of the secretion of interleukin 1-alpha was not inhibited. The development of sepsis after a lethal dose of lipopolysaccharide was significantly inhibited by pretreatment of the animals with tumor necrosis factor-alpha siRNA as 12 of 16 animal survived as compared to only one in eight for irrelevant siRNA. Unfortunately, the dose in this experiment is not reported (49).

In a recent report studying the effects of unformulated free siRNA, implanted tumors constitutively expressing luciferase showed a 40–50% decrease in luciferase activity after the tumor-bearing mice received an intraperitoneal, intravenous or subcutaneous injection of 3 μg of siRNA targeted against the reporter gene (50). Surprisingly, the route of administration had no effect on the magnitude of the knockdown unless the siRNA was injected intratumorally. Remarkably, this route of administration did not affect luciferase activity at all. In a therapeutic efficacy study daily intraperitoneal injections of 125 μg/kg/day (approx. 3 μg/injection) resulted in a 66% reduction in tumor volume over 16 days for siRNA targeted against vascular endothelial growth factor (VEGF), while reducing the tumor levels of VEGF by 70% as compared to animals treated with siRNA against luciferase. No adverse effects of treatment were noted. Interestingly, intraperitoneally administered siRNA against VEGF could no longer mediate a reduction in VEGF levels and siRNA against luciferase could no longer mediate a reduction in luciferase activity when the tumor expressed thrombospondin.

CONCLUSIONS

siRNA has made a remarkably rapid advancement from the first demonstration of its ability to invoke RNAi in mammalian cells in 2001 to the first reports on therapeutic efficacy in animal models within two years. The consistency between results from different laboratories and the magnitude of protein knockdown makes siRNA one of the most attractive tools in target identification and validation and functional genomics. An important area of research will be the development of clinically acceptable formulations of siRNA, ideally providing systemic, cell-specific, and efficient target cell delivery. It is expected that improvements in these formulations together with the shown possibility of disease modulation by siRNA provide future applications of siRNA as a new therapeutic modality.

ACKNOWLEDGMENTS

RMS is funded by the Dutch Cancer Society grant no. UU 2001-2185.

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