Antiviral Applications of RNAi

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Abstract RNA interference is a natural mechanism by which small interfering (si)RNA operates to specifically and potently down-regulate the expression of a target gene. This down-regulation has been thought to predominantly function at the level of the messenger (m)RNA, post-transcriptional gene silencing (PTGS). Recently, the discovery that siRNAs can function to suppress a gene's expression at the level of transcription, i.e., transcriptional gene silencing (TGS), has created a major paradigm shift in mammalian RNAi. These recent findings significantly broaden the role RNA, specifically siRNAs and potentially microRNAs, plays in the regulation of gene expression as well as the breadth of potential siRNA target sites. Indeed, the specificity and simplicity of design makes the use of siRNAs to target and suppress virtually any gene or gene promoter of interest a realized technology. Furthermore, since siRNAs are a small nucleic acid reagent, they are unlikely to elicit an immune response, making them a theoretically good future therapeutic. This review will focus on the development, delivery, and potential therapeutic use of antiviral siRNAs in treating viral infections as well as emerging viral threats.

Keywords RNAi · siRNA · PTGS · TGS · HIV-1

1 RNA Interference

RNA interference (RNAi), first described in plants and termed cosuppression (reviewed in Tijsterman et al. 2002), is a process in which double-stranded (ds)RNA induces homology-dependent degradation of mRNA (Montgomery...
RNAi is a process involving small interfering double-stranded (si)RNAs 21–22 bp in length, with 3′ overhanging ends that can induce a homology-dependent degradation of cognate messenger (m)RNA (Nishikura 2001). The generation of siRNA is the result of a multistep process that involves the action of RNase III endonuclease Dicer (Bernstein et al. 2001, 2003; Sui 2002; Fig. 1). The approximately 22-bp siRNAs that is processed by Dicer provide much of the specificity in the silencing process. However, the necessity for an exact sequence match in the sense strand of siRNA duplexes has been questioned, as single stranded antisense siRNAs can guide target RNA cleavage (Martinez et al. 2002) and as many as five mismatches in the sense strand RNA may be tolerated (Sumimoto 2003). In contrast, a single base pair mismatch relative to the target RNA on the antisense strand has been shown to significantly reduce siRNA-mediated message degradation (Hamada et al. 2002). Following the action of Dicer, the ~21-bp siRNAs are incorporated into the RNA-induced silencing complex (RISC), which identifies and silences by slicing the mRNAs complementary to the 21-bp siRNA through interactions with Argonaute 2 (Liu et al. 2004; Fig. 1). The specificity juxtaposed with potent suppression of target genes by siRNA has truly adopted RNAi as a standard methodology for gene specific silencing in mammalian cells.

Mechanistically, RNAi can suppress gene expression via two distinct pathways: transcriptional (TGS) and post-transcriptional (PTGS) gene silencing. PTGS involves siRNAs targeting of either mRNA or pre-mRNA, including intronic sequences in Caenorhabditis elegans and yeast (Bosher 1999). TGS involves silencing at the chromatin and was first observed when doubly transformed tobacco plants exhibited a suppressed phenotype of the transformed transgene. Careful analysis indicated that methylation of the targeted gene was involved in the suppression (Matzke 1989). TGS mediated by dsRNAs was further substantiated in viroid-infected plants and was shown to be due to RNA-dependent methylation of DNA (RdDM) (Wassenegger 1994). The observed TGS in viroid-infected plants contained viral promoters expressing integrated transgenes. Interestingly, these promoters became methylated at sites matching the small double stranded viral RNAs, and transcription of the viral promoters was suppressed as a result of these homologous viral RNAs entering the nucleus and inducing TGS (Wassenegger 1994, 2000), i.e., RNA directed suppression of gene expression at the promoter. In human cells, gene silencing induced by RNAi was initially thought to be restricted to action on cytoplasmic mRNA or RNA at the nuclear pore (Zeng 2002), similar to most reports in C. elegans and Trypanosoma brucei (Fire 1998; Montgomery 1998; Ngo 1998). To date, TGS has been found to occur in plants, Drosophila, and in Schizosaccharomyces pombe in centromeric regulation (Volpe 2002). Recently, TGS was reported to be operable in mammalian cells and appeared to rely on the delivery of the siRNA to the nucleus (Kawasaki et al. 2005; Morris et al. 2004a). However, the strict requirements of nuclear delivery may not be necessary if temporal factors are included in the analysis (Kawasaki and Taira
Fig. 1 Post-transcriptional RNAi in mammalian cells. Synthetic siRNAs or those generated by Dicer ex vivo can be transfected directly into cells using lipid-based transfection reagents or with siRNAs expressed from within the cell from lentiviral or other gene therapy-based vector systems (1). A cell can be stably transduced with a lentiviral vector that expresses siRNAs either from two independent promoters (U6, Pol III) or a single promoter driving the expression of a hairpin shRNA targeting a particular gene of interest (1). The vector-expressed siRNAs are probably bound by Exportin 5 and Drosha (2; Lee et al. 2003; Lund et al. 2004), and then get shuttled out of the nucleus and handed off to Dicer, which then cleaves the loop from the hairpin (3) producing the siRNA that is then loaded into RISC, ultimately leading to slicing of the target mRNA (4), essentially driving post-transcriptional gene silencing (PTGS).

The observed TGS in mammalian cells appears to involve DNA methylation, specifically DNMT1, DNMT3b (Kawasaki and Taira 2004), and DNMT3a (Jeffery and Nakielny 2004), as well as histone deacetylation, as the observed inhibition of gene expression was reversible with the addition of 5-azacytidine (5′Aza-C, 4 µM) and trichostatin A (TSA, 0.05 mM; Morris et al. 2004a).
2
Diversity of Viral Targets

Targeted suppression of human immunodeficiency virus (HIV)-1 has been achieved through siRNAs directed against HIV-1 tat and rev (Coburn 2002; Lee 2002; Novina 2002; Surabhi and Gaynor 2002), reverse transcriptase (Morris 2004; Surabhi and Gaynor 2002), trans-activating response region (TAR), and the 3′-untranslated region (UTR), Vif (Jacque 2002), as well as gag and the HIV-1 co-receptor CD4 (Novina 2002) and co-receptor CCR5 (Qin 2002; reviewed in Lee and Rossi 2004).

Viruses other than HIV-1 have also been successfully targeted by siRNAs in vitro with some success, including Semliki forest virus (SFV), poliovirus, dengue virus, influenza virus, hepatitis C virus, and many others (reviewed in Radhakrishnan et al. 2004). The fact that such a wide berth of varying viruses can be successfully targeted by siRNAs suggests that these nucleic acid molecules can be used to theoretically target virtually any emerging or present-day infectious agent. However, despite the excitement and the early proofs-of-principle in the literature, there are important issues and concerns about therapeutic application of this technology, including difficulties with efficient delivery, uncertainty about potential toxicity, and the emergence of siRNA-resistant viruses. In particular, certain viruses encode proteins that block one or more steps in the RNAi pathway (Bennasser et al. 2005; Hamilton et al. 2002; Johansen and Carrington 2001; Li et al. 2002; Llave et al. 2000; Mallory et al. 2001, 2002). Indeed resistance to siRNA occurs rather rapidly and is only contingent on a single nucleotide substitution (Gitlin 2002), and recently HIV-1 was shown to elude siRNA targeting by the evolution of alternative splice variants for the siRNA-targeted transcripts (Westerhout et al. 2005).

A possible way to circumvent such a conclusion in siRNA-mediated therapies for human viral infections could be to (1) design siRNAs to best fit targets from an extensive database of the variants in the particular target virus (Morris 2004) and (2) incorporate these best-fit siRNAs into a multiple anti-viral siRNA-expressing transgene vector. Undeniably, the multiplexing of several different siRNAs targeting different sites in the HIV genome along with non-essential cellular targets such as CCR5 should be utilized to harness the full potential of this mechanism in treating HIV-1 with siRNA technology. Alternatively, siRNAs designed to more conserved regions, such as to target viral intron/exon splice junctions, might also prove more resistant to the emergence of variant viral strains as the result of siRNA-mediated targeting.

3
siRNA Selection

There are many commercially available reagents as well as PCR-based methodologies (Castanotto and Rossi 2004) for use in the generation of synthetic
siRNAs. The usefulness of first generating and testing siRNA on a particular target prior to construction and generation of a vector system for the delivery and expression of a particular siRNA species (Morris 2004) cannot be overstated. Specific targeting of siRNAs is extremely important, as slight positional changes in the siRNA relative to the mRNA can have drastic effects on silencing (Holen 2002), indicating that the target mRNA secondary structure plays a role in the siRNA accessibility. Indeed not all siRNAs are functional, and a computational design or algorithm that provides 100% successful selection of efficacious siRNAs has not, to our knowledge, been developed. However, a set of common rules has begun to emerge from many of the studies done. SiRNAs in which the helix at the 5′-end of the antisense strand has a lower stability than the 3′-end of the siRNA are generally more effective than those with the opposite arrangement. A biochemical basis for the thermodynamic arrangement of effective siRNAs was provided by biochemical studies of the mRNA cleavage complex RISC in Drosophila embryo extracts, which showed unequal incorporation of the two strands of the siRNA into RISC (Schwarz et al. 2003). Strand biases could be manipulated by altering the thermodynamic stability of the terminal nucleotides in a way that precisely matched the rules that were derived from empirical studies. Finally, an examination of microRNAs (miRNAs), most of which produce RISC-like complexes containing only one strand of the precursor, showed the same pattern of thermodynamic asymmetry as did effective siRNAs (reviewed in Meissner 2001).

Another important factor in siRNA-mediated RNAi is based on cell type. siRNA-transfected cells that are actively dividing lose transcriptional silencing over roughly 96 h (Novina 2002; Tuschl 2002), possibly due to the cell division and subsequent loss of the required template mRNA (Holen 2002). In non-dividing cells, siRNA silencing has been retained long-term and correlates well with the presence of the mRNA target (Song 2003). Consequently, successful targeting of a desired transcript should involve prior attempts to model the siRNA accessibility to the template mRNA, similar to approaches employed with ribozyme and antisense RNA targeting (Scherr 1998). Furthermore, when targeting the RNA of a virus, conserved regions that cannot accommodate evolved point mutations should be preferentially selected. Certainly the sequence-specific ability of siRNA to inhibit gene expression suggests broad applications, including targeting of viral infections such as HIV-1. However, the sensitivity of siRNA to single base pair mismatches, coupled with extant data on the rapidity of evolution of drug resistance (Richman 1994) in the face of selective pressure, may limit the overall target selection in some viral infections.

4

Delivery of siRNAs to Target Cells

Once an siRNA or multiple siRNAs targeting a particular viral RNA have been designed and tested in vitro with transient-based transfection assays (Fig. 1),
it may prove necessary to express the siRNA from the context of the cell. The introduction of siRNAs into mammalian cells can be achieved through a variety of standard transfection methods (Fig. 1). The strength and duration of the silencing response delivered in the context of such transfection methods, however, is determined or limited (or both determined and limited) by several factors. On a population basis, the overall efficiency of transfection is a major determinant, which must be addressed by optimizing conditions. In each individual cell, silencing depends upon a combination of the amount of siRNA that is delivered and the potential of the siRNA to suppress its target (the potency). Even a relatively poor siRNA can silence its target provided that sufficient quantities are delivered. However, overloading the system with a high-concentration of siRNAs is likely to lead to undesired effects, including off-target suppression as well as the induction of a PKR response (Sledz et al. 2003). Indeed, there are innumerable methodologies available for expressing siRNAs from the context of the cell, including transient transfection of the synthesized or plasmid-expressed siRNA and stable expression of the particular siRNA by lentiviral vector delivery (Banerjea et al. 2003; Fig. 1).

Lentiviral vectors are emerging as one of the best candidates currently available for delivering and stably expressing short hairpin (sh)RNAs or siRNAs in target cells (Fig. 2). Lentiviruses, unlike retroviruses such as Moloney murine leukemia virus (MoMuLV), tend to preferentially integrate downstream of active promoters within the active transcriptional unit, potentially limiting their overall oncogenicity (Wu et al. 2003). Moreover, lentiviral-based vectors are capable of transducing non-dividing cells (Buchschacher 2000) and specifically targeting the nucleus. HIV-1-, HIV-2/SIV-, and feline immunodeficiency virus (FIV)-based lentiviral vectors are produced by co-transfecting vector, packaging, and envelope into producer cells, and collecting the resultant supernatants that contain the packaged vector 48 h later (Fig. 2). Lentiviral vectors are capable of stably transducing many cell types, including hematopoietic stem cells (Gervaix et al. 1997), integrating into the target genome, and expressing desired transgenes (Poeschla 1996; Price 2002; Quinonez 2002; Yam 2002). Lentiviruses have also been shown to cross-package one another (Browning 2001; Goujon 2003; White 1999). This observation has been carried over experimentally with HIV-1 and HIV-2 vectors being cross-packaged by FIV and capable of stably transducing and protecting human primary blood mononuclear cells from HIV-1 infection (Morris et al. 2004b). The cross-packaging of lentiviral vectors such as HIV-1 with an FIV packaging system offers a unique and possibly safer method for delivering anti-viral vectors to target cells in HIV-1-infected individuals. For instance, FIV-packaged HIV-1 or HIV-2 vectors reduce the likelihood of immune recognition, or seroconversion, due to exposure to HIV-1 structural proteins. Finally, lentiviral vectors can be specifically pseudotyped (Kobinger 2001; Sandrin 2003) or designed with a receptor-ligand bridge to target specific cell types (Boerger 1999).
Fig. 2 Production of lentiviral vectors. Lentiviral vectors are produced by (1) transfecting 293T producer cells with the lentiviral vector, packaging, and envelope plasmids. Next, the transfected cell transcribes the respective plasmids (2 and 3) subsequently producing the packaging co-factors (4) and vector RNA which is then packaged into the budding particles (4). The culture supernatants are collected 48–72 h later, and vector concentration is determined by tittering on target cells.

Therapeutically, the use of lentiviral or other stable integrating vector systems may not prove useful in the application of siRNAs in treating transient infections such as influenza or severe acute respiratory syndrome (SARS). One alternative is the use of cationic lipid complexes to systemically or locally de-
liver the viral or disease-specific shRNA or siRNA to the infected individual. Systemic delivery of siRNAs have been shown in mice and could be used to aid or augment the immune response during times of duress (Sioud and Sorensen 2003; Sorensen et al. 2003).

5 siRNA Challenges

Indubitably, one of the advantages of using siRNAs to treat emerging infectious agents such as viral infections is the relative ease of design, construction, and testing. The emerging field of RNAi—and siRNAs in particular—provides a potentially cost-effective and relatively quick methodology to treating some of the worlds most deadly emerging viral infections, such as Ebola and SARS, or to even deal with theoretical threats of smallpox or other viruses. Moreover, RNAi technology can also be used beyond the scope of human disease to treat agricultural, horticultural, and wildlife diseases. However, there are two important issues currently facing RNAi-mediated technologies that must be circumvented prior to the realization of RNAi in human therapeutics. These two constraints are the avoidance of off-target effects and the delivery of the siRNA to the target cell.

Steady progress has been made with regards to gene therapy-based delivery systems, specifically lentiviral-based vector systems. Regarding off-target effects, the use of siRNAs to target specific cellular or viral transcripts relies essentially on hijacking the endogenous RNAi machinery, of which we know very little, i.e., what is the potential for saturating the RNAi pathway. Indeed there is evidence that RISC can be saturated at least in the context of cultured cells (Pasquinelli 2002; Pasquinelli and Ruvkun 2002). Consequently, endogenous RNAi pathways appear to be susceptible to high concentrations of exogenous siRNA, suggesting that it will probably be imperative to not only quantitate siRNA-mediated silencing but to also monitor other genes in siRNA-treated cultures for untoward off-target effects. Indeed a thorough understanding of the mechanism(s) leading to nonspecific off-target effects as the result of siRNA treatment is essential before siRNAs or shRNAs can become realized in human therapies to treat viral infections.

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