Does RNA Interference Have a Future as a Treatment for HIV-1 Induced Disease?

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Abstract

RNA interference has recently emerged as an effective way to block the expression of specific messenger RNAs in eukaryotic cells. Using this approach, it has proven possible to block the replication of HIV-1 in cultured cells using small interfering RNAs targeted to viral sequences or to host messenger RNAs that encode factors critical for virus replication, such as the CCR-5 coreceptor. Unfortunately, the high sequence specificity of RNA interference, combined with the known tendency of HIV-1 to rapidly generate sequence variability, means that HIV-1 variants resistant to individual small interfering RNAs targeted to the viral genome arise rapidly. However, this problem may be circumvented by simultaneously targeting several essential HIV-1 sequences using RNA interference, or by targeting host genes that are essential for virus replication. Thus, RNA interference-based approaches have the potential to prove useful as novel treatments for HIV-1 induced disease, although the problem of how to efficiently deliver small interfering RNA expression vectors, or the small interfering RNAs themselves, to cells susceptible to HIV-1 infection* in vivo*, remains to be resolved. **(AIDS Reviews 2005;7:22-5)**

Key words

Gene therapy. HIV. Innate immunity. RNA interference.

The mechanism of RNA interference

RNA interference (RNAi) was first described in the nematode *C. elegans* and is now known to exist in all metazoan eukaryotes[1]. In *C. elegans*, introduction of long, double-stranded RNA (dsRNA) was found to result in the posttranscriptional silencing of homologous messenger RNAs (mRNAs) due to their cytoplasmic degradation[2]. Subsequently, it was demonstrated that RNAi required processing of the initial long dsRNA into ~22 base pair duplex RNAs, called small interfering RNAs (siRNAs), by the cytoplasmic RNase III enzyme Dicer. These siRNA duplexes have a characteristic structure, bearing an ~20 base pair double-stranded region flanked by 2 nt 3' overhangs. Subsequently, one strand of this siRNA duplex intermediate is incorporated into a protein complex called the RNA induced silencing complex (RISC), where it acts to guide RISC to mRNAs bearing a complementary sequence. Once bound to the target mRNA, a RISC component called Argonaut 2 cleaves the mRNA at the center of the complementary region and RISC then releases and goes on to cleave additional complementary mRNAs, i.e. RISC functions as an mRNA endonuclease whose specificity is programmed by the incorporated siRNA[1].

Although long dsRNAs function as efficient inducers of RNAi in *C. elegans*, as well as in plants and insects, they are ineffective in vertebrate cells[3] because of their ability to act as potent inducers of the interferon response, which induces a global inhibition of gene expression and can lead to cell death. Two approaches have been developed to circumvent this problem, both of which rely on the fact that dsRNAs of < 30 base pairs are inefficient inducers of the interferon response. One approach, first described by Tuschl and coworkers[4], involves the direct transfection into vertebrate cells of siRNA duplexes. An alternative approach, developed almost simultaneously by several laboratories[5,6], involves the transcription of a short hairpin RNA (shRNA) in the target cells. These shRNAs are basically siRNAs with one end closed by a terminal RNA loop, and these hairpins can be efficiently processed by Dicer to give the siRNA duplex intermediate, one

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strand of which is then incorporated into RISC. An advantage of shRNAs is that they can be constitutively expressed in target cells by transfection of expression plasmids, or more commonly, by transduction with a viral shRNA expression vector. In both cases, the ~60 nt shRNA is generally transcribed using an RNA polymerase III-dependent promoter, such as the H1 or U6 promoter, as polymerase III-dependent promoters, unlike RNA polymerase II-dependent promoters, do not induce terminal RNA modifications, such as capping and polyadenylation, that could interfere with the further processing of the shRNA by Dicer.

RNAi in animals is functionally closely related to a process in plants termed posttranscriptional gene silencing (PTGS) and considerable evidence exists indicating that PTGS is an important, innate, antiviral response in plants[7]. Thus, mutant plants that are unable to mount a PTGS response after viral infection are highly susceptible to viral pathogenesis. Moreover, many plant viruses encode proteins that act as inhibitors of the PTGS response, and viral mutants that express non-functional variants of these inhibitors are severely attenuated upon infection of wild-type plants[7]. These results suggested that RNAi might have the potential to act as a potent and specific inhibitor of viral replication in vertebrate cells. In this short review, I will discuss the use of RNAi as a strategy to inhibit HIV-1 replication in culture, and consider the potential of RNAi as a future treatment for HIV-1 induced disease.

The good news

There have been numerous reports describing the successful inhibition of HIV-1 replication in culture using both transient transfection of siRNA duplexes and stable expression of shRNAs. A wide range of viral sequences have been successfully targeted, including regions of the *gag* gene[8,9], the *pol* gene[8], the *vif* gene[10], the *tat* gene[11-13], the *rev* gene[13,14], the *env* gene[15], the *nef* gene[16], and the long terminal repeat (LTR)[10]. Consideration of the role of the various gene products encoded by these viral genes would suggest that mRNAs encoding the early regulatory proteins Tat and Rev might be particularly good targets for RNAi, as the production of the other late mRNAs encoding Gag, Pol, Env, and Vif is dependent on Tat and Rev function[17]. Moreover, because the viral Tat protein functions as a transcriptional transactivator of the viral LTR promoter, Tat strongly activates the expression of all viral mRNAs, including the Tat mRNA itself[17]. Indeed, a limited analysis has suggested that a Tat/Rev-specific siRNA is a more potent inhibitor of HIV-1 replication than several siRNAs targeted to *env*[18]. Nevertheless, siRNAs targeted to a wide range of sequences in the HIV-1 genome have been shown to be very effective in culture[8-16].

It should be noted that RNAi is believed to occur in the cytoplasm, so that pre-mRNAs are not effectively cleaved[1]. Thus, targets for RNAi located in the *gag* gene would likely only be cleaved in the context of the genome-length HIV-1 RNA that also functions as the mRNA for Gag and Gag-Pol, with all other viral mRNAs being unaffected. Conversely, the RNAi-induced cleavage of the 3'untranslated region (3'UTR) of an mRNA is as inhibitory as cleavage in the coding region. Therefore, siRNAs targeted to the *nef* gene would function not only as inhibitors of Nef expression, but also as inhibitors of all other viral mRNAs, as the *nef* coding sequence forms the 3'UTR for all other viral mRNAs.

An example of the inhibition of HIV-1 replication induced by an shRNA targeted to the viral *tat* gene is shown in figure 1. In this experiment, cells were transduced with a lentiviral shRNA expression vector that also encodes a blastocidin-resistance gene[12]. Blastocidin-resistant cells were then selected and challenged by infection with HIV-1. Inhibition of HIV-1 gene expression was monitored by Northern analysis of the infected cells (Fig. 1 A) and by quantification of the level of progeny virus release using an ELISA specific for the viral capsid (p24) protein present in the supernatant media (Fig. 1 B). As may be observed, this Tat-specific shRNA was able to essentially totally block HIV-1 replication in the transduced cells. Moreover, this inhibition was stable over a period of months and was effective in primary human CD4+ cells[12].

While the majority of RNAi studies relevant to HIV-1 have focused on target sequences located within the viral RNA genome, several investigators have also attempted to inhibit HIV-1 replication by downregulating mRNAs encoding host-cell factors that are critical for HIV-1 replication. These include the CD4 receptor[19], the CCR-5 and CXCR-4 coreceptors[12,20,21], and the host-cell proteins cyclin T1 and cdk9[22], which are required for the biological activity of the essential viral transcription factor Tat. While all of these host genes have been successfully targeted by RNAi, resulting in a substantial drop in the ability of cells to support HIV-1 replication, several of these cellular proteins also serve essential functions in the host and therefore are probably not good targets for RNAi in a clinical setting. The exception to this statement is CCR-5, a host-cell coreceptor that plays a critical role in the early stages of HIV-1 infection of humans[23]. Indeed, although HIV-1 variants that use the CXCR-4 protein as a coreceptor do arise late in infection in a minority of AIDS patients, all viruses recovered early in infection rely on CCR-5 for the infection of target cells *in vivo*. Importantly, humans that are naturally defective for CCR-5 expression exist, and these individuals are not only highly resistant to infection by HIV-1, but also entirely healthy;

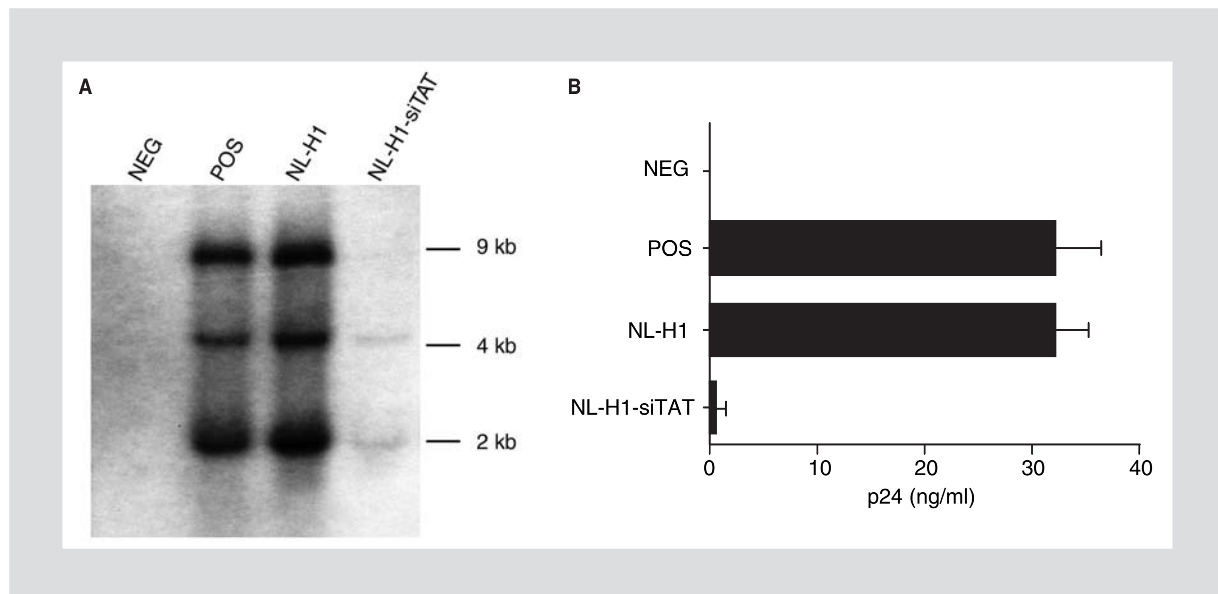

Figure 1. *Cells were infected with the NL-H1-siTAT lentiviral shRNA expression vector, or the NL-H1 control vector, and transduced cells selected based on antibiotic resistance. These cells were then challenged with HIV-1 and viral replication analyzed at 48 hr after infection by Northern blot (panel A) or release of progeny virions (panel B). POS, non-transduced cells; NL-H1, cells transduced with a control lentiviral vector; NL-H1-siTAT, cells transduced with a lentiviral vector expressing an HIV-1 tat gene specific siRNA* (modified from reference 12).

i.e. CCR-5 is dispensable for human well-being[23]. This host-cell gene product is therefore a potentially very promising target for ablation using RNAi.

The bad news

An important characteristic of RNAi is that this process is highly sequence specific. Thus, the expression of target mRNAs can be efficiently inhibited, yet even quite similar non-target mRNAs normally remain unaffected. Unfortunately, this high level of specificity means that slight mutation of the RNAi target sequence can make a virus partially or even fully resistant to RNAi.

As predicted from the above, passage of an initially homogenous HIV-1 virus isolate, derived from a proviral clone, in the presence of an inhibitory siRNA can result in the rapid selection of resistant HIV-1 variants. Thus, in the case of an siRNA targeted to the essential *tat* gene, a resistant viral variant bearing a 1 nt missense mutation at the center of the targeted *tat* sequence was detected after 25 days of passage and this variant rapidly grew out to become the dominant viral form[24].

A second study used an siRNA targeted to the nonessential *nef* gene of HIV-1, thus permitting a wide range of resistant viruses, including several viral deletion as well as point mutants, to appear[25]. One resistant HIV-1 mutant is particularly interesting, as this virus fully maintained the *nef* sequence targeted by the siRNA used, but contained a 1 nt substitution mutation 7 nt 5' to the targeted sequence. Remarkably, this single point mutation was shown to stabilize an RNA secondary structure that sequesters much of the targeted *nef* sequence into an RNA stem-loop[25]. It has been previously demonstrated that the siRNA incorporated into RISC cannot effectively target an mRNA sequence located in a secondary structure[26]. Thus, HIV-1 is able to use a range of different mutations, both inside and outside the targeted viral sequence, to escape from RNAi-mediated inhibition. Given that patients are normally infected with a swarm of different but closely related HIV-1 variants, that rapidly arise in all infected patients due to the inaccuracy of viral reverse transcription, we can confidently predict that HIV-1 variants resistant to all individual siRNAs would preexist in patients prior to any treatment with the siRNA in question.

Problems and solutions

Despite this serious caveat, RNAi remains a potential future treatment for HIV-1 induced disease. At least two strategies to overcome the problem of viral resistance can be proposed. One strategy is to target several highly conserved HIV-1 sequences simultaneously using several different siRNAs. In the case of poliovirus, which also rapidly evolves to escape from inhibition by single siRNAs, this approach has proven to be highly effective at preventing the appearance of resistant viruses, at least in culture[27]. A second approach is to target a host gene essential for HIV-1 replication, the

most obvious one being CCR-5. Host genes do not show the sequence variation seen in viruses, and based on studies using drugs that block the ability of CCR-5 to function as a coreceptor, it is known to be difficult, albeit not impossible, for the virus to evolve the ability to dispense with CCR-5[23].

The second issue that stands in the way of using RNAi as an antiviral strategy in a clinical setting is delivery. One potential approach (i.e. the introduction of artificial siRNA duplexes by some form of *in vivo* transfection) is obviously very challenging due to issues of transfection efficiency, appropriate tissue targeting, the stability of siRNAs *in vivo*, and finally, the question of how long the induced RNAi response would last. Nevertheless, several companies are focused on using various forms of chemical modification of siRNAs in order to solve these technical problems. The alternative gene therapy approach, while also clearly technically challenging, currently seems more feasible, especially as several groups have shown that the stable expression of shRNAs from lentiviral vectors introduced into the germ line of mice has no evident deleterious consequences[28,29]. Moreover, human CD34+ hematopoietic progenitor cells that were transduced with a lentiviral anti-Rev shRNA expression vector, and allowed to differentiate into mature macrophages or T-cells (in the latter case in SCID-hu mice) remained able to block HIV-1 replication effectively when challenged[30]. Therefore, lentiviral vectors expressing an anti-CCR-5 shRNA, or a range of shRNAs targeted to important HIV-1 sequences, have the potential to inhibit HIV-1 replication *in vivo* over the long term without any deleterious consequences to the host. At this stage it would seem to be important to generate transgenic mice that express shRNAs specific to several target sequences present in some form of pathogenic mouse virus, such as the mouse retrovirus Murine Leukemia Virus, to provide proof-of-principle that shRNA can provide effective protection against retrovirus-induced pathogenesis. If this desirable result is indeed obtained, then it will be worthwhile to consider moving RNAi targeted to HIV-1 into a clinical setting.

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