RNA interference (RNAi) is a natural mechanism in cells that suppresses or silences the expression of aberrant or foreign genes. This activity is being developed as a potential antiviral therapeutic strategy. Studies in vitro, and some in vivo, appear to show the feasibility of using RNAi to treat virus infection. Therapeutic use of RNAi seems to be promising when directed against viruses that cause localized acute infections in accessible target cells. Therapeutic strategies using RNAi against viruses that cause chronic infections, such as HIV, hepatitis B virus, or hepatitis C virus, are more difficult to design, but studies have begun to address identifiable problems. Two clinical trials using RNAi have recently been initiated—one phase II trial against respiratory syncytial virus and a phase I trial against HIV. It will be of much interest to see whether nucleic acid therapies can offer another route to treating viral infection.

Introduction

A phase I clinical trial was approved in May 2007 by the US Food and Drug Administration to evaluate RNA interference (RNAi) as a nucleic acid therapy for HIV-1 infections [1]. This trial is the first to employ a lentivirus vector for transduction into hematopoietic stem cells (HSCs) that later differentiate into different immune cells. This vector design is the first tested that targets, genetically, a combination of three sites in HIV replication. It is also the first employing RNAi in its therapeutic design using short hairpin RNA (shRNA) directed against the Tat/Rev site of the HIV genome. The other two nucleic acid constructions placed into this vector include a ribozyme designed to decrease host cell CCR5 expression by degrading mRNA for CCR5 and an oligonucleotide decoy that binds and sequesters the TAR protein synthesized in HIV-infected cells [1–6]. Gene therapy, previously focused on correction of DNA defects, stopped after deaths occurred in two different human trials [7]. Strategies for gene therapy have since been directed at inhibiting the effects of aberrant or noncellular genes. HIV trials employed mechanisms associated with expression of ribozymes or antisense RNA [8,9]. Neither approach has been as successful as anticipated for any targeted therapy [7]. The recognition during the 1990s of RNAi as a natural, biological means of silencing specific genes led to its widespread use as a laboratory tool to examine gene function. The antiviral potential of RNAi was quickly realized and tested.

RNAi

RNAi is a pathway of intracellular mechanisms involved in regulating gene expression. RNAi provides cellular protection against invasive nucleic acid, such as viral genomes, and the effects of abnormal genes. RNAi, first observed in plants [10], was found to be conserved and operative in other eukaryotes including humans [10,11•]. Although short interfering RNA (siRNA) is used extensively in vitro, some success manipulating the RNAi process in vivo for potential antiviral use has occurred. Subsequent reports suggest adequate feasibility to plan phase I clinical trials [12]. Several reviews have focused on different aspects of using RNAi against viral infection and for other clinical applications [4–6,11•,13,14•,15,16•]. Some of them provide detailed descriptions and figures about the mechanisms involved in RNAi cellular pathways [6,14•,16•].

RNAi makes use of double-strand RNA (dsRNA) species found transiently in the cell. Events associated with RNAi occur in the cytoplasm. The interference mechanism employs dsRNA in siRNA form or microRNA (miRNA) and is usually around 19 to 25 basepairs (bp), but no more than 30 bp, in length. The size limitation imposed on the small RNA species helps to avoid initiation of immune responses, especially interferon (IFN), which arise in response to the presence of dsRNA longer than 30 bp in length [11•,16•] contributing to the pathogenic processes of some viruses. During RNAi, short RNA associates with a complex of host-cell enzymes. These include the RNA-induced silencing complex (RISC) and argonaute 2.
(AGO2), which initiate degradation of target RNA synthesized in the cell. siRNA are dsRNA perfectly matched in sequence; miRNA are imperfectly matched dsRNA.

Basically, siRNA or miRNA are generated by endogenous cleavage of longer precursor cellular dsRNA with a cellular enzyme, Dicer, an RNase III [4–6,11•,13,14•,15,16•] (Fig. 1). Synthetic siRNA may also include hairpin structures (shRNA) that may function like siRNA after cleavage by Dicer. Alternatively, siRNA already at working length are introduced into the cell and bypass cleavage to act against intracellular copies of mRNA from infectious agents such as viruses. The aggregate of proteins and enzymes comprising RISC attaches to either siRNA or miRNA. It is thought that one of the enzymatic functions contained in RISC cleaves one of the strands of siRNA or miRNA, usually the sense strand, activating RISC in the process. The activated complex then finds and binds to a target messenger RNA (mRNA), containing a complementary nucleotide sequence that matches the strand remaining in RISC. The binding interferes with the expression of target mRNA. If the binding is perfect, as would be seen for siRNA, then target mRNA is cleaved by one of the enzymes in RISC and releases activated RISC to be used again. If the binding is not exact, similar to what is seen for miRNA, the activated complex disrupts mRNA expression by blocking translation.

RNAi is a natural means of cellular gene regulation but is adaptable for use as a defense mechanism. Several characteristics of the RNAi pathway are particularly attractive for development as a therapy against infectious agents such as viruses. The mechanism provides a way to act on targets specific to each viral replication cycle [11•] that cannot be reached easily with current medicinal therapeutics [14•,15,16•]. This approach, involving natural host cell components, would potentially reduce the toxicity to host cells that can occur during treatment with conventional drugs. Because RNAi treatment would be designed from knowledge of the targeted viral sequence, it is possible to generate and test prospective siRNA quickly. The interference would not require targeting of just the functional areas of a viral protein but would allow targeting to be anywhere on viral mRNA. Design strategies could proceed as soon as sequence data of the target genome were obtained, even if the activity encoded by the target region of the genome was not known [11•,14•]. This feature clearly allows increased flexibility in design and number of targets available for RNAi agents. The release of activated RISC allows it to be recycled for repeated use due to the stability and efficacy of the enzymes in RISC [17]. The presence of siRNA introduced exogenously is short lived [17], again reducing the possible toxicity of the treatment.

Despite the promise of treatment with RNAi, there are challenges involved in designing an RNAi therapy that can work in vivo. These include delivery of the product to specific target cells in efficacious amounts, decreasing the potential of unintentional binding of siRNA or miRNA to highly homologous off-target sequences in the cell genome (which may alter cell functioning detrimentally), and the development of “escape” mutations in the target sequence, especially if the target is a viral
polymer was injected retro-orbitally into mice. Alternately, the same influenza genes encoding the nucleoprotein and enza used different strategies to deliver siRNA targeting delivery and specificity. Two studies [24,25] using influenza virus (SARS-CoV) evaluated other variables in siRNA influenza and severe acute respiratory syndrome coronavirus infections in mice, which acquire these viruses by the same transmission route as humans.

Mice inoculated intravaginally with HSV-2 virus develop infection and apparent disease, sometimes paralytic and lethal. A candidate siRNA targeting the HSV-2 DNA binding protein was administered vaginally in a lipid mixture used normally for in vitro transfection [22•]. Previous evaluation of this delivery agent showed it was efficacious without eliciting an inflammatory response or IFN production. Of 20 mice treated with the siRNA 2 hours before challenge and 4 hours after challenge with two LD_{50} of HSV-2, only 25% died compared with 75% in the control groups (n = 17). Also, those that survived cleared infection by day 11, suggesting that siRNA treatment may have decreased HSV pathogenicity and lessened or prevented viral shedding. During this short period, there was no evidence of escape mutants to siRNA treatment.

Mice inoculated intranasally with RSV show maximal virus growth in the lung 5 to 6 days later and increased respiratory rate, mucus secretion, inflammation, and bronchoconstriction. Mice inoculated with parainfluenza virus showed similar effects. Mice inhaling siRNA directed against the RSV P protein diluted in transfection reagent or in tissue culture medium, before or with virus challenge, displayed no infection [23•]. If siRNA was given after challenge, the infection was reduced, and there was a faster cure rate. Because RSV and parainfluenza virus are sometimes identified in the same clinical sample, concurrent administration of two different siRNA, one to each virus, was also examined. Mice pretreated with the siRNA mixture, then challenged with a mixture of both viruses, were protected. No disease or signs of viral infection was evident in harvested lung tissue. IFNα and γ in tissue were also absent.

Reports of RNAi treatment of the respiratory viruses, influenza and severe acute respiratory syndrome coronavirus (SARS-CoV) evaluated other variables in siRNA delivery and specificity. Two studies [24,25] using influenza used different strategies to deliver siRNA targeting the same influenza genes encoding the nucleoprotein and polymerase A. In one study [24], siRNA in a cationic polymer was injected retro-orbitally into mice. Alternately, shRNA targeting the influenza proteins was placed into a lentivirus vector and transfected intratracheally or retro-orbitally. In another study [25], siRNA diluted in phosphate buffered saline was administered intravenously in the tail vein, followed by challenge with an influenza/siRNA mixture intranasally. Both strategies appeared to work prophylactically and after infection, increasing survival, reducing lung viral titers and decreasing weight loss during infection.

The SARS-CoV study [26] was performed in rhesus macaques, which showed disease characteristics similar to, but more severe than, infected humans during SARS outbreaks. Two siRNA were tested against spike protein and nonstructural viral protein genes and had exact sequence homology to a laboratory strain and to 100 sequenced global isolates. No homology to human genome sequences was noted. Because SARS-CoV replication and pathology occur inside the lung, an effective carrier was sought that would reach into lung tissue. Three groups of macaques were treated intranasally with siRNA in a glucose-water carrier before, during and after infection with different dosing regimens. All groups displayed some symptoms of disease. The siRNA-treated animals generally had lower fevers or nearly normal temperatures. One siRNA, linked with fever suppression, also decreased antiviral IgG levels, which developed up to 9 days after the controls. There was milder diffuse alveolar damage, lower virus replication, and fewer infected cells in deep lung tissue.

RNAi intervention with Japanese encephalitis virus and West Nile virus infection [27] demonstrated the complexity of delivery of siRNA to other tissues. Both flaviviruses travel to the brain to cause meningoencephalitis in humans. Infected mice show similar disease. The viral envelope gene, highly conserved between both viruses, was targeted by siRNA or shRNA in a lentivirus vector. Optimizing with different lipid delivery agents and doses intracerebrally, a single dose of siRNA was given 6 hours after four LD_{50} of virus. All mice survived; 60% of mice receiving siRNA 18 hours after infection survived indefinitely. No evidence of virus replication in the brain was noted in the surviving mice up to 21 days later, by histopathology or virus culture. A siRNA was also developed that suppressed both Japanese encephalitis virus and West Nile virus infections in vivo.

**Chronic infections**

RNAi therapy for chronic infection presents different challenges. Chronic infection can establish at sites peripheral to the initial site of infection. Persistently infected tissues may shed virus continuously or have potential for oncogenesis. Viruses causing chronic sequelae often produce indistinct, mild, acute disease symptoms that resolve quickly. The damage from chronic infection may result from a long-term immune response to continual presence of viral antigen. Pharmaceutical treatment of chronic infections reduces disease by suppressing viral
replication; RNAi would act similarly. Use of RNAi to suppress chronic infection requires that siRNA or shRNA reach [28,29] and have potential for prolonged expression in appropriate target cells [30]. Long-term expression may be facilitated using shRNA delivered in a vector rather than siRNA, which exists transiently [7].

Targeting viral genes
Initially, RNAi strategy for chronic infection focused on reducing acute viral replication to reduce the development of pathogenesis during the chronic phase. Expression of RNAi agents after delivery was monitored.

Activity of two shRNA/plasmid constructs against hepatitis B virus (HBV) was assessed after cotransfection with HBV genomes into mice [31]. These shRNA were expressed in the liver, showing that the plasmid reached appropriate organs and cells after tail vein injection. HBV infection was suppressed but not inhibited, evidenced by a substantial decrease in all replicative forms of HBV, hepatitis B surface antigen, and the number of hepatocytes containing HBV antigen. A similar study [32] using shRNA against regions conserved among the HBV genotypes yielded similar results. In a different report, HBV was inoculated with siRNA into mice [33]. Chronic HBV infection was observed as long as 120 days after infection. Compared with controls, siRNA treatment reduced viral load to levels similar to antiviral treatment with lamivudine and adefovir.

Interferon knockout mice were infected with Coxsackievirus B3 (CVB3), then treated 6 and 14 hours after infection by tail vein or by intraperitoneal transfection with siRNA against a site coding for CVB3 proteinase 2A protein [34]. This protein can cleave dystrophin in myocardial cells [35], potentially leading to dilated cardiomyopathy after acute viral infection resolves. Histologic examination showed that 50% of the heart cells expressed the siRNA after transfection. No virus was detected in the heart and lungs of treated animals but was present in control mice. Virus in the liver was significantly reduced. These data suggest that RNAi optimization could lessen inflammation responding to active replication of virus in myocardial cells to decrease heart damage.

Targeting cellular genes
Another approach using RNAi to intervene with chronic infection is to suppress a cellular gene in susceptible cells needed for virus replication, without causing permanent harm. A common target is the cellular receptor used for viral entry. An in vitro study [36] examined inhibition of both CVB3 and adenovirus infection by reducing expression of their common cellular receptor, coxsackie-adenovirus receptor. Like CVB3, adenovirus can establish persistent infection in the heart [36]. Cells received shRNA/vector instead of siRNA partly because cells of cardiac origin are difficult to transfect. Infection of HL-1 cells, a cardiac muscle cell line, with CVB3 was inhibited in a dose-dependent manner. shRNA silencing of coxsackie-adenovirus receptor was less protective against challenge with adenovirus. Downregulation of the receptor in primary rat neonatal cardiomyocytes occurred around 6 days after receiving shRNA. If challenged with either virus, less virus replication occurred. Challenging earlier did not reduce viral yields.

A cellular gene (miR22) encoding a protein that interacts with the 5’ noncoding region of hepatitis C virus (HCV) was targeted [37]. This protein is not found in all cell types but is present in Huh7 cells in which HCV constructs can replicate. The area of the HCV genome to which miR22 binds is conserved in all six HCV genotypes. RNAi targeting of miR122 reduced HCV protein expression in these cells, implying that successful inhibition of this protein reduced the amount of HCV mRNA synthesized in infected cells.

Targeting oncogenic and immune-modulating potential
siRNA targeting the cellular FAS gene in mice was used to inhibit induction of fulminant hepatitis [38]. FAS mediates apoptosis. Administered by hemodynamic tail vein injection, siRNA was present in the liver within 24 hours and prevented liver cell necrosis, inflammation, and development of fibrosis. The protection was transient; the reduction of FAS protein and its mRNA peaked by 10 days, returning to normal within 20 days. Other FAS-related genes were not affected. Although the model did not address viral infection, it was suggested that suppressing FAS might reduce the severity of chronic liver damage induced by viral infection.

RNAi reduction of oncogenesis was demonstrated in a model of human papillomavirus (HPV) tumor formation [39]. Tumors form when CaSKi cells, which contain 600 copies of HPV16, are implanted into legs of nude mice. siRNA designed to target HPV E6 mRNA, encoding a protein with oncogenic activity, was inoculated into either the peritoneal cavity or subcutaneously into tumor sites daily for 5 or 12 days or only twice, on day 5 and 10. For all treated groups, tumor growth was smaller, and expression of HPV E6 protein was much lower, sometimes almost undetectable. Apoptosis of tumor tissue was higher compared with controls.

The feasibility of using siRNA against hepatitis G virus, a flavivirus, in marmosets was examined [40]. The animals were inoculated intravenously with siRNA/cationic liposome targeting a site in the untranslated region of the virus that differs from that of HCV, also a flavivirus, by two nucleotides. In a 3-day siRNA treatment protocol, marmosets were infected in the liver on day 2 with hepatitis G virus in plasma from another infected monkey. Treated monkeys showed delayed infection, as did the controls initially, but had lower plasma viral loads for 6 months. Both groups induced IFN, which would suppress the virus, although possibly by two different routes—one through local induction, one by specific induction.
Application of RNAi to HIV Infections

HIV replication has been studied extensively in vitro using RNAi [4–6,11•,15]. Therapeutically, design of RNAi strategy may be more complex as much is known about the challenges of HIV-1 treatment. Acute infection is mild and indistinguishable from several other infections. Individuals may not know they have acute HIV infection. Therefore, RNAi therapy would likely focus on chronic infection. During acute infection, the virus travels to many reservoirs and cell types throughout the body to establish persistent and latent infection that may not be detected for several years. Delivery of RNAi therapeutic agents would need to reach these sites. Also, the make-up of quasispecies carried by the infected individual constantly changes, arising from small sequence variation that could affect the efficacy and design of nucleic acid–based therapy. The virus replicates very quickly and easily selects escape or resistant mutants in response to antiretroviral therapy. A complication is that many subtypes of HIV-1 found globally have distinct sequence variation, and the virus recombines easily among subtypes to form circulating recombinant forms. Finally, there is no animal model that completely encompasses all these features, making in vivo evaluation of new therapeutic agents, such as RNAi, difficult.

Target and Design

Several RNAi therapeutic studies targeted host-cell receptors for the virus. An early study evaluated the use of siRNA against the cellular CD4 gene as well as the viral gene encoding the GAG protein [41]. Although the data showed this approach to be promising when siRNA was transfected 24 hours prior to HIV infection, the authors suggested that CCR5 might be a better target. Subsequent studies successfully targeted CCR5 expression, as the protein seems to be dispensable in humans [2,3,42] and HIV tends to infect cells with this receptor more during early HIV infection. Downregulation of the host CCR5 gene was shown in cell lines carrying siRNA, shRNA, or ribozyme delivered by a lentivirus vector leading to a reduction of HIV viral load. One study incorporated a second shRNA against the CXCR4 gene into the same vector and showed this biphasic shRNA-vector could also work successfully in peripheral blood mononuclear cells [43]. Of interest is a report of the ability of the Tat protein made in HIV-infected cells to be able to circumvent RNAi by suppressing Dicer activity [44]. It was suggested that shRNA might not be as effective in HIV-infected cells because it would need to be processed by Dicer but that short precut siRNA delivered to the cell would bypass the block. The vector and constructs used in the phase I HIV RNAi trial [1–3] are a lentivirus capable of infecting cells that are not actively dividing. The siRNA target is the Tat/Rev region of the HIV genome [2]. The sequence of this construct should allow it to be active in binding spliced and unspliced mRNA found in the infected cell so that it may silence HIV genes during two different replication stages.

Subtype infection

Two studies examined the potential of RNAi to suppress infections with different subtypes [45,46•]. shRNA against conserved regions of subtype C gag [45] and subtype B gag, Rev, and Vif [46] was tested against infection with clinical isolates of different geographic origin and phenotypic characteristics. The silencing activity of the subtype C shRNA to gag varied depending on the clinical isolate [45]. The activity of shRNA against subtype B Vif was the most successful, able to inhibit infection with subtype B strains from China and the United States as well as a recombinant B/C strain [46•]. Sequence analysis indicated that target mRNA had to be highly conserved within the binding region of the shRNA but could tolerate some variation at the 5’ or 3’ ends, emphasizing the importance of this information when designing siRNA or shRNA.

Escape mutations

Reports of escape mutations in HIV treated by RNAi reinforced data observed with RNAi of other virus infections [4,6,21]. The appearance of escape mutants is not surprising, especially after knowledge gained during development and use of antiretroviral drugs. Studies indicated that the replicating virus developed the mutations and not the shRNA [18]. Resistance developed after a few weeks in vitro [18,19]. The types of mutations varied. Most mutations were point mutations [18–20,47]. Other escape mechanisms included a deletion of 106 bp that removed the binding site targeted by siRNA and the acquisition of mutations sequentially [19]. Also observed was development of mutations outside of the siRNA binding site so that the target virus developed a different folding secondary structure causing the target site to be inaccessible to siRNA [20]. In a very thorough study, each site in a 17-bp target was altered systematically to evaluate binding by siRNA [47]. The data, similar to those reported for subtype binding [46•], indicated that the vulnerable bases causing loss of siRNA binding efficacy were in the center of the target sequence or were flanking the center region. More tolerance was shown at the extreme ends.

Conclusions

It is likely that therapy using RNAi will be tailored to each target virus [11•]. Currently, RNAi design seems to be less complicated for viruses causing only acute infections. The HSV-2 [22•] and RSV infection [23•] models show that specific tissues can be targets for siRNA treatment in nonviremic infections. They suggest that primary tissue sites that are accessible and are of mucosal origin can be treated with siRNA efficaciously to suppress acute infection when delivered in simple transfection or physiologic
medium. RNAi may be an effective microbiocide. A phase II trial testing RNAi of RSV infection, announced in June 2007 [16•], reinforces the potential of siRNA as an inhaled therapeutic agent. The influenza, SARS-CoV, and flavivirus models demonstrated possible benefits of using RNAi therapy following viral exposure if delivery of the agents can be optimized. They show that design of the RNAi sequence to target regions with highly conserved sequence might also increase potential for use against related viruses [23••,27,36] with wider geographic distribution.

RNAi therapy models against chronic infection address the issues involved with stability and delivery of agents for RNAi. Because the initial site where the virus enters the host may be different from the location where chronic infection establishes, it is important to identify nontoxic compounds/solutions that could selectively deliver agents for RNAi to tissues difficult to reach, like heart muscle, without significant toxicity [34,40]. The studies suggest that RNAi could be used to intervene with chronic disease by either siRNA or shRNA delivered in nonreplicating viral vectors. The prolonged efficacy of RNAi that suppress processes associated with chronic disease such as oncogenesis and inflammation [38,40] is implied.

Designing RNAi therapy for chronic HIV infection will not be simple, and feasible animal models are not available. The presence of escape mutations and sequence variation in in vitro HIV infections indicates that problems faced in conventional antiretroviral therapy also exist for RNAi therapies. The use of combination therapy in RNAi [2], as for current antiretroviral strategy, may be a viable strategy to circumvent this problem. Long-term expression of agents of RNAi in multiple reservoirs and cells is still a challenge. The attraction of RNAi against HIV therapeutically is that it is a natural biological mechanism that may have much less associated toxicity in the face of life-long treatment. Also, it may be less expensive than conventional antiretroviral therapy following viral exposure if delivery of the agents for RNAi to tissues difficult to reach, like heart muscle, is feasible animal models are not available. The presence of escape mutations and sequence variation in in vitro HIV infections indicates that problems faced in conventional antiretroviral therapy also exist for RNAi therapies. The use of combination therapy in RNAi [2], as for current antiretroviral strategy, may be a viable strategy to circumvent this problem. Long-term expression of agents of RNAi in multiple reservoirs and cells is still a challenge. The attraction of RNAi against HIV therapeutically is that it is a natural biological mechanism that may have much less associated toxicity in the face of life-long treatment. Also, it may be less expensive than current therapy [1]. It will be of great interest to see how the clinical trials using RNAi fare.

Disclosure
No potential conflict of interest relevant to this article was reported.

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