RNA interference: a potential novel therapeutic combating HIV-1 in the central nervous system

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Summary

RNA interference (RNAi) is a conserved process by which eukaryotic cells protect their genomes utilizing small, double-stranded RNAs to degrade target RNAs. This occurs in a sequence-specific manner and is different from the interferon effect of larger double-stranded RNAs. Post-transcriptional gene silencing by these nucleic acids can lead to degradation of either cellular or viral RNAs. It has been recently shown that double-stranded, small interfering RNAs (siRNAs) of 21 to 25 nucleotides can be transfected into relevant cells to target specific RNAs. In addition, utilizing hairpin motifs, siRNAs can be expressed intracellularly using molecular therapeutic vectors. This potent approach has been utilized to both inhibit pathogens, including viruses, as well as to dissect cellular molecular mechanisms via a potent knockout effect. At this time in the HIV-1-pandemic, one of the remaining, most enigmatic, and still vitally important areas of HIV-1 pathogenesis occurs in the central nervous system (CNS). HIV-1-induced encephalopathy remains difficult to treat in the developing world and in parts of the developed world, even in the era of highly active anti-retroviral therapy. As such, novel approaches which could lead to intracellular immunization, and life-long resistance against HIV-1 encephalopathy would be of important impact worldwide. Thus, we now seek to combine our background in molecular therapeutics and RNAi with our long-standing interest in HIV-1 neuropathogenesis to target the CNS using siRNAs.

Key words: RNA interference • siRNA • therapeutics • HIV-1

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HIV-1-INDUCED ENCEPHALOPATHY

A series of devastating clinical conditions in the central nervous system (CNS) of certain untreated or non-virally suppressed HIV-1-infected individuals may be caused by infection of cells in the brain parenchyma. These clinical syndromes, which lead to a wide variety of CNS signs and symptoms, are not fully understood. Patients with AIDS-dementia complex (ADC) are often characterized by apathy and inattentiveness, as well as slowing of motor movements and in coordination. Intellectual dysfunction based on alterations in memory, language, and problem solving are found in later stages of the disease. In addition, ADC left untreated will frequently continue until a vegetative state is reached. Of note, clinical-lem solving are found in later stages of the disease. In based on alterations in memory, language, and prob-
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HIV-1 frequently infects the CNS of individuals soon after seroconversion. Studies have demonstrated clearly that microglia and monocytes/macrophages are major cellular reservoirs for highly productive HIV-1 infection in the CNS. As well, several recent studies have demonstrated HIV-1 provirus and low-level replication, in vivo, within microvascular endothelial cells (MVEC), astrocytes, and neuronal elements, utilizing both immunocytochemistry and in situ polymerase chain reaction. Several studies have also demonstrated limited replication of SIV and HIV-1, in vitro as well as in vivo, within MVECs, HIV-1 replication has been evaluated in some detail within human fetal and adult astrocytes, both in vitro and in vivo. (see below). These studies suggest a restricted form of HIV-1 replication in astrocytic cells, characterized mainly by expression of multiply spliced HIV-1 mRNA, which encode certain viral regulatory gene products (e.g. Rev, Tat, and especially Nef). Certain populations of neurons are infectable by HIV-1 and SIV both in vitro and in vivo. Although recent trials utilizing combinations of reverse transcriptase inhibitors and viral protease inhibitors, entitled highly active anti-retroviral therapy (HAART), have shown important efficiency in altering HIV-1 replication in vivo; these approaches may have several weaknesses in targeting HIV-1 reservoirs within the CNS. First, the penetration of some of these compounds across the BBB is not always efficient, leading to lower CNS concent-
trations of certain of these anti-lentiviral drugs compared with levels in the peripheral blood. This is due to effects of the P-glycoprotein of the BBB on protease inhibitors (see below) and to active efflux pumps altering nucleoside-analog reverse transcriptase inhibitor CNS levels (for a review see reference). As well, the intracellular concentrations of these agents, and in some cases active metabolites, are poorly described for the intracellular milieu of various CNS-based cells (possible partial “sanctuary sites”).

Recent studies have demonstrated low levels of cryptic replication of HIV-1 and latent virus in CD4+ T cells and macrophages in patients on virally suppressive HAART leading to residual disease and viral reservoirs. Of note, a few cases have demonstrated worsening of CNS symptoms in patients on triple anti-HIV-1 therapy, which dramatically decreased viral levels in the peripheral blood. Also, “low-level” or indolent forms of HIV-1-induced encephalopathy may occur in some patients on virally suppressive HAART. Nevertheless, most patients with ADC on suppressive HAART have gained stability of ADC signs and symptoms, and some patients have cognitive improvement. Of interest is a study in which it was demonstrated that HIV-1 RNA in cerebrospinal fluid is produced from both the peripheral blood and the brain parenchyma in infected individuals. Finally, as brain and retina are difficult tissues to obtain in healthy HIV-1-infected individuals on virally suppressive HAART, our analysis of HIV-1 persistence and latency in semen, which we have recently shown may have a different molecular mechanism than in peripheral blood, represents an initial demonstration of HIV-1 residual disease behind a true blood : tissue barrier.

As mentioned above, although we are two decades into the HIV-1 epidemic, the exact mechanisms which lead to ADC remain somewhat obscure. Hypotheses suggesting both direct and indirect mechanisms, based on retroviral replication in the CNS, have been proposed to account for ADC. Several HIV-1 gene products, especially Tat and the envelope glycoprotein (gp120), have been suggested as putative neurotoxins. In addition, neurotoxins secreted by HIV-1 monocytes/macrophages and microglia have also been suggested as causative moieties in ADC. These include: tumor necrosis factor α, interleukin 1, tissue growth factor β1, platelet activating factor, and arachidonic acid metabolites. Nitric oxide over-production may have an effect on ADC, although this is not demonstrable in all systems. Apoptosis has also been implicated in neu-
ronal loss in ADC. Data have been reported which suggest that interactions between HIV-1-infected macrophages and astrocytic cells are necessary to allow expression of neurotoxins. Neuronal damage, in this model, is based on over-stimulation of N-methyl-D-aspartate receptors on neurons and subsequent increased intracellular calcium levels\textsuperscript{38, 131}. Recent data have suggested that apoptosis (or programmed cell-death) of CD8\textsuperscript{+} and/or CD4\textsuperscript{+} T lymphocytes may play a role in AIDS pathogenesis (for a review see reference\textsuperscript{93}). In addition, recent preliminary studies have suggested that apoptosis of neuronal elements may also lead to the neuronal loss noted in patients with ADC\textsuperscript{2, 4, 5, 42, 46, 62, 95}. A study has confirmed and extended data on increased apoptosis of neurons, astrocytes, and MVEC in patients with ADC compared with non-demented HIV-1-infected individuals\textsuperscript{111}. The mechanism involved in inducing CNS-based apoptosis during HIV-1 infection may be based on effects induced by HIV-1 gp120 \textsuperscript{2, 20, 88, 89}, Vpr\textsuperscript{55, 60} or Nef\textsuperscript{135}. A study has also suggested that CNS apoptosis may be secondary to the expression of the c-kit proto-oncogene, stimulated by HIV-1 Nef, utilizing \textit{in vitro} analyses of an astrocytic cell-line\textsuperscript{135}. Our laboratories have recently demonstrated the quantitative importance of HIV-1 virions and cell-free viral-specific proteins on inducing human neuronal apoptosis. As such, novel approaches to inhibit ADC, even in the era of HAART, remain quite necessary. A robust molecular platform, such as RNA interference (RNAi), may be quite useful to develop in this respect (see below).

**RNA INTERFERENCE**

RNAi is a newly-described natural biological phenomenon mediated by small interfering RNA (siRNA) molecules which target specific mRNA for degradation by cellular enzymes. RNAi has become a method of choice for studying gene function, especially in mammalian systems. With proof-of-concept studies already presented against a wide variety of human pathogens and several innovative methods of delivering the siRNA to a wide variety of primary cells available, the role for siRNA as a potential therapeutic strategy is becoming clearer\textsuperscript{45, 49, 56, 58–60, 71, 87, 90, 104, 129}.

The short length of siRNA (21-23 nucleotides) and the high degree of homology required within the target region of the cognate transcript usually lead to the selective destruction of the desired transcript. This specificity is the most attractive feature of RNAi as an antiviral approach. However, the high degree of homology also creates an obvious drawback because viruses are notoriously sloppy in this replication and this leads to the production of mutated progeny. This feature of viral replication is already known as a mechanism which helps viruses to escape immune surveillance or inhibition of drugs. Since the effect mediated by siRNA is severely affected or even eliminated when the target region of the cognate transcript differs by even a single nucleotide, genetic variations in viruses may be expected to impact on the potential of siRNA as a therapeutic agent. As noted, RNAi is the well-known phenomenon of post-transcriptional gene silencing (PTGS) that has been conserved in lower eukaryotes, plants, and mammals. RNAi is initiated when double-stranded RNA (dsRNA) is present in the cytoplasm (often a consequence of viral infection), suggesting that plants and animals share a conserved antiviral mechanism leading to specific destruction of non-self dsRNA. dsRNA is cleaved by the RNaseIII-like enzyme, Dicer, to 21-26 nucleo-
tides siRNA which then mediates destruction of the cognate transcript. In mammalian cells, RNAi functions independently of the IFN-induced pathways. Dicer is a member of the RNaseIII family of nucleases that specifically cleave dsRNA and is evolutionarily conserved in worms, flies, plants, fungi, and mammals. The enzyme has a distinctive structure, which includes a helicase domain and pre-mRNAs are, in contrast, resistant to RNAi. RNA-dependent RNA polymerase (RdRp) is another enzyme critical for PTGS. RdRp is encoded by viruses and several eukaryotes. They have non-homologous sequences but possess similar function, including copying mRNA templates and intercellular spread of the amplified sequences. There are indications that RNA amplification by RdRp is not essential in human cells for RNAi. However, multiple rounds of cleavage do take place. It has been shown that cross-linked siRNA duplexes also mediate RNAi, indicating that complete unwinding of the siRNA helix is not necessary for RNAi activity in vivo. In vitro synthesized siRNA is now routinely transfected into a wide variety of cell-lines with the aid of a variety of lipid reagents. This approach limits the use of siRNA-mediated PTGS to cell-lines only. The cost of siRNA is considered to limit the number of targets that can be tested. In order to overcome the above factors, a variety of different approaches have been employed. Each of these approaches involves driving siRNA expression under RNA pol III promoters. These include the human U6, mouse U6, and the human H1 and the human 7SK promoters. The pol III promoter’s normal products are small, highly structured, and are found in a variety of subcellular compartments, so they are ideal for expressing siRNA. The most abundant RNAs transcribed from a pol III promoter are the U6 small nuclear RNA, which plays a crucial role in the processing of premRNA; the H1, an RNA component of RNase P; and the 7SK RNA, a negative regulatory of RNA polymerase II elongation factor TEF. Of the above promoters, the H1 promoter is being increasingly used. Transcripts produced specifically from U6 promoters apparently are regulated by cellular mechanisms, which ensures that with increasing dose of transfected genes, the transcript levels and half-life are dramatically decreased. siRNA can be cloned as a hairpin or the sense and antisense strands can be transcribed from two different pol III promoters in the same vector. Using lentiviral, retroviral, and adenoviral vectors allows RNAi analysis to be performed in a wide variety of cells (including primary cells). The effects of siRNA thus delivered persist for a much longer duration. Lentiviral vectors with H1 promoters driving siRNA expression have been successfully used for long-term gene silencing. Silencing against enhanced green fluorescent protein (EGFP) was found to be dose dependent and occurs as early as 72 h post infection and persists for at least 25 days. Cloning the H1-siRNA cassette in the U3LTR has the advantage of duplicating during the replication of the virus, thus presenting an even more potent effect. Adenoviral vectors carrying the H1-siRNA expression cassette have also been used effectively for long-term gene silencing.

In vitro synthesized siRNAs have been used without any kind of cloning for PTGS to cell-lines only. A somewhat problematic method has been employed in post-natal rats, involving rapid injection of a large volume of physiological solution into the tail vein. This high-pressure delivery of siRNA enabled specific silencing of target genes EGFP or luciferase in several different organs. In vitro synthesized siRNAs have antiviral activity in cell cultures. This was initially demonstrated for HIV-1 by several groups. If siRNAs are expressed from inside the cells, rather than being administered to the outside, the cells become largely refractory to subsequent HIV-1 infection. siRNA targeted to various regions of the HIV-1 genome inhibit infection by specifically degrading genomic HIV-1 RNA, thereby preventing formation of viral complementary DNA intermediates. siRNAs have also been shown to inhibit HIV-1 infection of permanent cell-lines and primary CD4+ T cells. The infection seems to be inhibited at two points in the viral life-cycle: after fusion and before reverse transcription and also during transcription of viral RNA from integrated provirus. Treatment of HIV-1-infected activated CD4+ T cells with a fluorine-derivatized siRNA that is resistant to RNase A yielded similar inhibition of HIV-1 infection. The derivatized siRNA has the advantage of being delivered without complexing to a lipid reagent and in the presence of serum. siRNA duplexes targeted against the essential Tat and Rev regulatory proteins encoded by HIV-1 can specifically block Tat and Rev expression and function. These same siRNAs can effectively inhibit HIV-1 gene expression and replication in cell cul-

In vitro
infection of CXCR4+ or CCR5+ U87-CD4+ cells by co-receptor expression effectively blocked the acute influencing CD4 expression. The suppression of HIV-1 mediated the silencing of these genes specifically, without affecting cells by the corresponding siRNA. siRNA mediates the silencing of these genes specifically, without influencing CD4 expression. The suppression of HIV-1 co-receptor expression effectively blocked the acute infection of CXCR4+ or CCR5+ U87-CD4+ cells by X4 (NL4-3) or R5 (BaL) HIV-1 strains. The infection was inhibited regardless of the multiplicity of infection. A similar study was conducted in human peripheral blood T lymphocytes. A lentivirus-based vector was used to introduce siRNA against CCR5. There was a 10-fold inhibition of CCR5 expression over a period of 2 weeks without any influence of CXCR4 expression. This resulted in substantial protection for the lymphocyte populations from CCR5-tropic HIV-1 virus infection, decreasing infected cells by 3- to 7-fold, while only a minimal effect on decreasing infection by a CXCR-5-tropic virus was observed.

In another approach to block HIV-1 infection, siRNA against the chemokine receptors have been used. These siRNA effectively eliminated cell surface expression of the chemokine receptor and thereby prevented HIV-1 entry. Suppression of chemokine receptors was detected 48 h post-infection. CXCR4 and CCR5 expression was blocked in 63% and 48% of positive cells by the corresponding siRNA. siRNA mediated the silencing of these genes specifically, without influencing CD4 expression. The suppression of HIV-1 co-receptor expression effectively blocked the acute infection of CXCR4+ or CCR5+ U87-CD4+ cells by X4 (NL4-3) or R5 (BaL) HIV-1 strains. The infection was inhibited regardless of the multiplicity of infection. A similar study was conducted in human peripheral blood T lymphocytes. A lentivirus-based vector was used to introduce siRNA against CCR5. There was a 10-fold inhibition of CCR5 expression over a period of 2 weeks without any influence of CXCR4 expression. This resulted in substantial protection for the lymphocyte populations from CCR5-tropic HIV-1 virus infection, decreasing infected cells by 3- to 7-fold, while only a minimal effect on decreasing infection by a CXCR-5-tropic virus was observed.

Our laboratories have recently demonstrated that siRNAs to a major HIV-1 chemokine co-receptor, CXCR4, can be used to inhibit cell-to-cell fusion during HIV-1 infection with X4-tropic virus. These studies have suggested that the inhibitory qualities of this siRNA are more potent against cell-to-cell fusion than free-virus-to-cell infection. The molecular mechanisms behind these phenomena may be based on the differences in viral infection and interactions with differing densities of CXCR4 expressed on the target cell surface, between cell-free and cell-associated viral infection.

In addition, recent data from our laboratories have also demonstrated our ability to generate siRNAs to inhibit neurotropic strains of HIV-1. Targeting the HIV-1 virus itself, especially in the gp41, Tat, Rev open-reading frames, we were able to demonstrate potent inhibition of many of the major neurotropic strains commonly evaluated in the laboratories and likely in primary neurotropic isolates. These studies targeted mainly R5-tropic viruses, the major viral strains found in the central nervous system of infected individuals. As such, these two approaches targeting CXCR4 on neurons and viral-specific RNAs from neurotropic strains now allow us to interdict in HIV-1 infection in CNS-based cell-types by both attacking a cellular co-factor as well as the virus itself. In addition, we also now have data that APJ, a CNS-based co-receptor for HIV-1, can also be targeted using the RNA interference approach.

In summary, we hypothesized that HIV-1 infection in the CNS would be a relevant target for RNAi approaches. The increasing maturation of the RNAi field allows us to rationally design techniques to both inhibit HIV-1 replication and neuronal dysfunction/death in the CNS.

REFERENCES


