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Reversal of HIV-1 Latency with Anti-microRNA Inhibitors

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Abstract

Human immunodeficiency virus type 1 (HIV-1) latency is achieved when host cells contain integrated proviral DNA but do not produce viral particles. The virus remains in resting CD4 T-lymphocytes, evading host immune surveillance and antiviral drugs. When resting cells are activated, infectious viral particles are produced. Latency is critical for the survival of all HIV-1 strains in vivo. Recently, it has been reported that a cluster of cellular microRNAs (miRNAs) enriched specifically in resting CD4+ T-cells suppresses translation of most HIV-1-encoded proteins in the cytoplasm, sustaining HIV-1 escape from the host immune response. Complementary antisense miRNA inhibitors block the inhibitory effect of miRNAs and drive viral production from the resting T-lymphocytes without activating the cells. Therefore, inhibition of these HIV-1-specific cellular miRNAs is of great therapeutic significance for eliminating the HIV-1 reservoir in HIV-1-infected individuals receiving suppressive highly active antiretroviral therapy (HAART).

Keywords

HIV-1; latency; microRNA; Tat; Rev; viral reservoir; HAART

Introduction

MicroRNAs (miRNAs) are short RNAs of 20~22 nucleotides that participate in the regulation of various biological processes in numerous eukaryotic lineages, including plants, insects, vertebrates, and mammals (Ambros & Chen, 2007; Bartel, 2004). The expression of many miRNAs is usually specific to a tissue or developmental stage, and changes in miRNA expression patterns have been observed in the development of many human diseases (Ambros & Chen, 2007; Calin & Croce, 2006; Farh et al., 2005; Lim et al., 2005). Mature miRNAs are generated from primary miRNAs by sequential digestion via the Drosha and Dicer proteins (Gregory et al., 2004; Grishok et al., 2001; J. Han et al., 2004; Hutvagner et al., 2001; Lee et al., 2003). Mature miRNAs are assembled into a miRNA-induced silencing complex (miRISC) of which Dicer and Argonaute 1–4 (Ago1–4) are essential components. The mature miRNA typically interacts with a complementary sequence in the 3' untranslated region (3'-UTR) of target mRNAs by partial sequence matching, resulting in degradation of the mRNA and/or translational inhibition (Bartel, 2004). A defense role for cellular miRNAs against viral infection has been demonstrated in mammalian cells (Lecellier et al., 2005; Yeung, Benkirane, & Jeang, 2007). Further, cellular miR-17-5p and miR-20a play a role in regulating the expression of the histone acetyltransferase Tat cofactor, p300/CBP-associated factor and

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subsequently, HIV-1 replication (Triboulet et al., 2007). In addition, virus-derived miRNAs can participate in the regulation of host and/or viral gene expression (Bennasser, Le, Benkirane, & Jeang, 2005; Klase et al., 2007; Ouellet et al., 2008; Pfeffer et al., 2004).

Cellular miRNAs play a vital role in maintaining HIV-1 latency

It was recently discovered that cellular miRNAs inhibit HIV-1 expression in primary resting CD4⁺ T-cells via interactions with the 3'-end of HIV-1 RNA, thereby contributing to viral latency (Huang et al., 2007). Almost all spliced or unspliced HIV-1 mRNAs share the same 3'-UTR during translation. Cellular miRNAs bind to the 3'-UTR of HIV-1 mRNAs and can inhibit the translation of almost all HIV-1-encoded proteins, including Tat and Rev, key regulators of viral gene transcription and viral RNA translocation. The GFP-expression vectors containing 1.2-kb fragments from the 3'-UTR of various HIV-1 genomes were transfected into resting CD4⁺ T-lymphocytes. Expression of GFP from these vectors was reduced in resting primary CD4 T-lymphocytes compared to a control vector lacking the viral sequences, indicating that the element(s) responsible for miRNA inhibition was contained within this 1.2-kb region. Five putative miRNA binding sites (for mir-28, -125b, -150, -223, and -382 binding) were identified via deletion analysis of the 1.2-kb region, and mutation of these sites significantly neutralized the inhibition, indicating that the binding sites were sufficient for inhibition of GFP expression in resting CD4⁺ T-cells (Huang et al., 2007). Further, 2'-O-methyl-oligoribonucleotide antisense inhibitors of these miRNAs significantly counteracted the inhibitory effects of cellular miRNAs on GFP expression from the 1.2 kb fragment-containing expression vector. The neutralization of the inhibitory effects of these five miRNAs by their corresponding antisense inhibitors resulted in increased viral production, as assessed by p24 antigen levels in cell culture supernatant following transfection of resting CD4⁺ T-lymphocytes with an infectious clone of HIV-1. Application of individual inhibitors only modestly affected viral production, whereas the combination of all five inhibitors significantly increased the production of both HIV-1 proteins and infectious particles (Huang et al., 2007). Further, the combined miRNA inhibitors increased HIV-1 infectious particles at least 10-fold compared with the undetectable viral particles induced by a control miRNA inhibitor in resting CD4⁺ T-cells isolated from several HIV-1-infected individuals receiving suppressive highly active antiretroviral therapy (HAART) (Huang et al., 2007). Importantly, the miRNA binding sequences identified in the 3'-UTR of various HIV-1 strains are relatively conserved, and the inhibition of HIV-1 expression, although variable in degree, was observed in many strains of HIV-1 including R5 (YU2, and JR-CSF), X4 (NL4-3 and LAI), and dual-tropism (89.6) viruses. These cellular miRNAs have also been shown to inhibit viral strains directly isolated from resting CD4⁺ T-cells of HIV-1-infected individuals (Huang et al., 2007). Thus, the mir-28, -125b, -150, -223, and -382 cellular miRNAs likely participate in maintaining or establishing the latency of all strains of HIV-1 in resting CD4⁺ T-cells.

The miRNA-mediated control of *Tat* and *Rev* expression is key for HIV-1 latency

HIV-1 Tat protein binds to the Tat-activation region (TAR) at the initial site of viral RNA transcripts. Several cellular factors such as p-TEFb, CDK9, and cyclin T1 also bind to TAR in a Tat-dependent manner. Tat-activated CDK9 then hyper-phosphorylates the C-terminal domain of RNA polymerase II, subsequently increasing the processivity of the transcription elongation complex (Herrmann, Gold, & Rice, 1996; Zhou et al., 2000). Inefficient expression of Tat protein in the resting CD4⁺ T-cells therefore plays a key role in maintaining HIV-1 latency. In the absence of Tat, RNA polymerase II remains hypo-phosphorylated. In addition, Tat can bind to histone acetyltransferases (HATs) p300/CBP, p300/CBP-associated factor, and hGCN5 (Benkirane et al., 1998; Hottiger & Nabel, 1998; Marzio, Tyagi, Gutierrez, & Giacca, 1998). Tat-recruited histone acetyltransferases may induce the hyperacetylation of histones in

nucleosomes proximal to long-terminal repeats, subsequently activating viral transcription (Lusic, Marcello, Cereseto, & Giacca, 2003). Many incomplete viral transcripts have been found in HIV-1-infected resting CD4⁺ T-cells, suggesting a poor processivity of RNA polymerase II (Lassen, Ramyar, Bailey, Zhou, & Siliciano, 2006). Overexpression of Tat in HIV-1-infected resting CD4⁺ T-cells significantly increases the proportion of full-length transcripts and induces the generation of HIV-1 viral particles, indicating the importance of Tat insufficiency in maintaining HIV-1 latency (Lassen et al., 2006). Alternatively, multiply spliced and unspliced HIV-1 RNAs have been found in latently infected resting CD4⁺ T-cells isolated from HIV-1-infected individuals receiving suppressive HAART. These RNAs mainly exist in the nucleus (Hermankova et al., 2003; Lassen, Bailey, & Siliciano, 2004; Lassen et al., 2006). It is well established that Rev plays a key role in the transport of unspliced RNAs from the nucleus to the cytoplasm via interaction with an Rev-responsive element (RRE) in the envelope region. If unspliced RNA is retained in the nucleus because of Rev insufficiency, splicing will gradually occur. The concentration of Rev correlates with the concentration of unspliced RNA in the cytoplasm and with generation of HIV-1 viral particles. Therefore, the shortage of Rev in these cells could also contribute to HIV-1 latency (Pomerantz, Seshamma, & Trono, 1992; Pomerantz, Trono, Feinberg, & Baltimore, 1990).

Because both spliced and unspliced HIV-1 RNAs can be detected in resting CD4⁺ T-cells (Chun et al., 2003; Furtado et al., 1999; Lassen et al., 2006; Patterson et al., 2001; Zhang et al., 1999), latency and escape of immune surveillance may be achieved by inhibition of viral antigen production through the recruitment of resting cell-enriched cellular miRNAs. When cellular miRNA function is blocked by antisense miRNA-directed inhibitors, more Tat and Rev will be synthesized to facilitate the generation of viral transcripts and the transporting of viral transcripts into the cytoplasm. Simultaneously, Tat inhibits the activity of miRNAs (Bennasser et al., 2005). As such, a positive feedback loop occurs that facilitates more viral RNA synthesis and viral production (Fig. 1). Conversely, a negative feedback loop comes into play when activated CD4⁺ T-cells switch to resting status and CD4⁺ T-cell-enriched miRNAs gradually increase. Based on this hypothesis, a novel therapeutic method could be developed to induce all the replication-competent viruses from HIV-1 latently infected resting CD4 T-lymphocytes.

Concerns regarding anti-miRNA inhibitors to reverse HIV-1 latency

Before using miRNA-specific antisense inhibitors to reverse HIV-1 latency in resting CD4⁺ T-lymphocytes, several aspects should be considered. One important concern is that other mechanisms besides miRNA inhibition also play an important role in maintaining HIV-1 latency. It has been reported that several cellular factors also contribute to the inefficient viral transcription in resting CD4⁺ T-cells (Blankson, Persaud, & Siliciano, 2002; Y. Han, Wind-Rotolo, Yang, Siliciano, & Siliciano, 2007; Kim et al., 2006; Williams et al., 2006). Therefore, there could be two possibilities regarding the population of latently-infected CD4 T-lymphocytes. The first possibility is that some latently-infected cells do not generate any viral RNA because of severe inefficiency of cellular transcriptional factors in these cells. The activation of cells by mitogen or cytokine(s) will activate the transcription from these proviruses. Another possibility is that the resting cells harboring transcriptionally active HIV-1 RNA represent all the replication-competent virus-harboring cells isolated from HIV-1-infected individuals receiving suppressive HAART. The activation of cells by mitogen or cytokine(s) will only significantly enhance the transcription from these proviruses. These two possibilities remain to be clarified. Because a significant amount of short and incomplete viral RNAs exist in the nucleus of infected resting CD4⁺ T-cells isolated from HIV-1-infected individuals receiving suppressive HAART and because exogenous Tat can significantly induce viral production (Hermankova et al., 2003; Lassen et al., 2004; Lassen et al., 2006), the second possibility is highly likely. If confirmed, manipulation of RNA transcripts could induce the

viral production and exposure of the infected cells to immune surveillance of the host. Therefore, it will be quite valuable to manipulate the expression of these miRNA in resting CD4⁺ T-lymphocytes.

Another important concern is that it is necessary to optimize the combination of miRNA inhibitors to induce replication-competent HIV-1 viruses present in resting CD4 T-lymphocytes. Each cellular miRNA is capable of regulating hundreds of targets (Bartel & Chen, 2004; Linsley et al., 2007). Until recently, there has been no information regarding the cellular function of most identified HIV-1 latency-related miRNAs. However, it has been reported that mir-223 participates in granulocytic differentiation (Fazi et al., 2005). Therefore, systematic application of antisense miRNAs in vivo may be toxic. In the case of the HIV-1 3'-UTR, almost all the viral proteins are regulated by at least five cellular miRNAs. Single miRNA inhibitors can only counteract the inhibition from one miRNA. In such cases, the other four major miRNAs remain functional, thereby maintaining the inhibition on viral RNA. Therefore, it is not surprising that individual miRNA inhibitors did not significantly rescue viral production. When miRNA inhibitors were used in combination, all the major latency-related cellular miRNAs were inhibited. As a result, viral production was rescued. Nevertheless, it is still possible that inhibition of HIV-1 viral production may not require all five miRNAs. Only two or three miRNA binding sites, in the absence of anti-mir-223 inhibitor, are sufficient to inhibit viral protein synthesis. To investigate possible synergetic effects of these miRNA inhibitors on the HIV-1 production, various combinations of miRNA inhibitors should be further examined and optimized.

Finally, effective introduction of antisense inhibitors of miRNAs into resting CD4⁺ T-lymphocytes in vivo could present a technical challenge. In invertebrates and plants, RNAi can spread systemically to silence gene expression distant from the initiation site. The genes *SID-1* and *SID-2* play a key role in this process (Winston, Molodowitch, & Hunter, 2002; Winston, Sutherland, Wright, Feinberg, & Hunter, 2007). However, RNAi does not spread systemically in mammals. A *SID-1* homolog was recently identified in human cells (Wolfrum et al., 2007). It plays an important role in systemic delivery of siRNAs that are conjugated with cholesterol and contained within HDL (Wolfrum et al., 2007). Unfortunately, *SID-1* expression is low in leukocytes (Wolfrum et al., 2007). Nevertheless, liposome-based nanoparticles were recently generated for systemic and efficient delivery of siRNA into leukocytes in vivo (Peer, Park, Morishita, Carman, & Shimaoka, 2008; Peer, Zhu, Carman, Lieberman, & Shimaoka, 2007). Therefore, it is possible to formulate a nano-liposome which contains antisense miRNA inhibitors. If the nano-liposome has been made, the delivery efficiency into resting CD4⁺ T-lymphocytes isolated from HIV-1-infected individuals receiving suppressive HAART should be examined. If these reagents work well in cell culture, further in vivo work (animal experiment) for systemic delivery should be studied.

Concluding remarks

HIV-1 latency in resting CD4 T⁺ lymphocytes may result from suppression of Tat and Rev expression by a cluster of cellular miRNAs. Antisense inhibitors of the cellular miRNAs can remove the inhibition of Tat and Rev expression and drive viral production from the resting T-cells. As a result, latently infected resting CD4 T-lymphocytes could be exposed to immune surveillance. If optimal combinations of these inhibitors could be effectively delivered into resting CD4 T-lymphocytes in vivo, eradication of viral reservoirs may be possible for HIV-1 patients undergoing HAART treatment.

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Figure 1. Mechanisms how anti-miRNA inhibitors activate HIV-1 latency in resting CD4 T-lymphocytes

Anti-miRNA inhibitors block the inhibitory effect of cellular miRNAs on Tat and Rev translation. As a result, more Tat and Rev migrate into the nucleus and bind to TAR or RRE respectively. Tat recruits CDK9 and cyclin T1 to bind with TAR. CDK9 subsequently hyperphosphorylates the C-terminal domain of RNA polymerase II in the region of HIV-1 promoter and increase its processivity. Consequently, more HIV-1 transcripts will be generated and Tat and Rev protein will be translated. Rev in the nucleus facilitates the export of unspliced viral RNA transcripts from nucleus into cytoplasm. Moreover, Tat protein accumulated in the cytoplasm inhibits the function of either RISC or miRNAs and therefore will augment the inhibitory effect of anti-miRNAs on the function of miRNAs.