RESEARCH ARTICLE

Inhibition of HIV-1 fusion with small interfering RNAs targeting the chemokine coreceptor CXCR4

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RNA interference (RNAi) is an evolutionarily conserved process by which plants and animals protect their genomes utilizing small, double-stranded RNAs to degrade target RNAs in a sequence-specific manner. Post-transcriptional gene silencing by these moieties can lead to degradation of both cellular and viral RNAs. It has recently been shown that double-stranded, small interfering RNAs (siRNAs) of 21–25 nucleotides can be transfected into relevant cells to target specific RNAs. This approach was utilized to inhibit human immunodeficiency virus type I (HIV-1) infection in human cells. siRNAs with homology to a motif in the mRNA that encodes for the HIV-1 chemokine coreceptor CXCR4 was utilized. Complementary studies via immunofluorescence microscopy and fluorescence-activated cell sorting demonstrated downregulation of CXCR4 from the surface of cells transfected with the specific siRNAs. As well, siRNAs without sequence homology to CXCR4 were used as controls and demonstrated no downregulation of CXCR4. siRNAs targeted to another chemokine coreceptor, APJ, showed specificity for downregulation of APJ but had no effects on CXCR4.

Transfections with siRNAs targeting CXCR4 mRNA were shown to inhibit HIV-1 envelope fusion, which is relatively resistant to most viral inhibitors targeting chemokine coreceptors. The specificity of this effect was demonstrated by the inhibition of fusion by CXCR4-tropic and dual-tropic (CXCR4 and CCR5) envelope glycoproteins from HIV-1 on CXCR4+ indicator cells, but the lack of effects by siRNAs targeting CXCR4 mRNA on dual-tropic HIV-1 envelopes in CCR5+ indicator cells utilizing these fusion assays. Interestingly, siRNAs targeting CXCR4 selectively inhibited CXCR4-tropic cell-free virus infection of human cells but at only modest levels as compared to cell:cell fusion. siRNA may be a potential molecular therapeutic approach to alter a cellular cofactor critical for infection of human cells by relevant strains of HIV-1. The targeting of a cellular cofactor, rather than the HIV-1-specific mRNAs or genomic RNA, holds promise as the rapid mutational ability of the HIV-1 genome may obviate the potential clinical use of RNAi directly against this virus. Gene Therapy (2004) 11, 1703–1712. doi:10.1038/sj.gt.3302339; Published online 12 August 2004

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Introduction

Recent studies have shown that diverse ‘micro-RNAs’ of approximately 22–25 nucleotides are critical in animal development, including mammals.^{1–5} Small temporal RNAs have been well studied in the control of precise developmental events in nematodes.⁶ Of note, noncoding RNAs make up 98% of the genome in humans and are now known to act as critical factors in complex regulation cascades of all eukaryotic cells.^{7,8} As such, these small RNAs have been undergoing extensive investigations for use in understanding the function of multiple cellular proteins as well as potential antimicrobial therapy against various intracellular pathogens.^{9–17}

Originally discovered by accident in studies of *Caenorhabditis elegans*, double-stranded RNAs were also

shown to alter inheritable characteristics in these organisms.⁴ This was quickly followed by demonstration of post-transcriptional gene silencing (PTGS) by these moieties in plants and then other animals, and is now commonly referred to as RNA interference (RNAi).¹⁴ It has been clearly demonstrated that double-stranded RNA, with homology to a specific gene sequence, can act as a ‘trigger’ for PTGS. Unique aspects of this process are both the sequence specificity, leading to targeted mRNA degradation, and the requirement that of small interfering double-stranded RNAs (siRNAs) 21–25 nucleotides be produced via cleavage of longer double-stranded RNAs, proceeding to ongoing sequence-specific attack of target RNAs.¹⁸ These studies have demonstrated that this mechanism does not involve interferon, as longer (ie greater than 30 nucleotides) double-stranded RNAs would induce. Unlike RNAi, interferons lead to global, nonspecific effects on many cellular and viral RNAs.³ The cleavage of targeted RNAs in RNAi occurs via RNase III-like enzymes, entitled ‘Dicer’. The siRNAs that are produced function in an siRNA protein complex or RNA-induced silencing complex (RISC), containing ATP and a helicase for unwinding of siRNA strands. In certain non-mammalian systems, it has been clearly shown that single-stranded siRNAs bind to the target

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mRNA and then lead to the formation of double-stranded RNAs by RNA-dependent RNA polymerase (RdRP). The new double-stranded RNAs are then cleaved by Dicer into further 21–25-nucleotide siRNAs. This last step acts as an amplification or catalytic process, which increases the intracellular efficiency of RNAi.^{19,20} Of note, though, this amplification step has not been demonstrated in mammals, and recent initial data suggest that it may not take place in either mammals or *Drosophila*.²¹

siRNAs, which seem to always require short 3' overhangs,^{3,22} were first shown to be useful in altering gene product expression by transfection into invertebrates. These siRNAs can be chemically synthesized or created via *in vitro* transcription systems, and maintain their activity after introduction into mammalian cells.^{2,3} Recent studies also suggest that siRNAs may be quite active in mammals when introduced *in vivo*.¹⁵

Of importance, the primary function of some of these evolutionarily conserved double-stranded RNAs may be in the protection of the host genome from transposons and viruses.^{2,16,17} The first demonstrations that RNAi acted against viruses were performed in plant systems and then recently in *Drosophila*.²³ Based on these studies, it has become clear that siRNAs may be useful in developing molecular therapeutics for human viral pathogens.

Of note, human immunodeficiency virus type I (HIV-1) is a possible target for development of RNAi to halt initial infection of human cells or spread of the virus after infection, with the ability to target multiple aspects of its complex life cycle.^{13,24} One potential point of attack using this newly developed technology would be the site of virion binding and entry of critical human target cells.

HIV-1 enters most human cells by initial binding to the high-affinity CD4 receptor, as well as binding of the envelope glycoprotein (gp120) to critical chemokine coreceptors, especially CXCR4 and CCR5.^{25,26} CXCR4 belongs to the family of seven transmembrane G-protein-coupled receptors.^{27–31} These coreceptors transduce signals via heterotrimeric G-proteins. CXCR4 has been clearly identified as one of the major coreceptors for HIV-1 cell entry *in vivo*.²⁶ As well, recent studies with knockout mice of CXCR4 have demonstrated that this molecule plays an important role in immunomodulation, organogenesis, hematopoiesis and cerebellar neuron migration.^{32–34}

The discovery of chemokine coreceptors for HIV-1 tropism demonstrates the phenotypic diversity conferred by coreceptor specificity. Viral isolates obtained in HIV-1-infected individuals during asymptomatic stages generally are CCR5-tropic.³⁵ In contrast, a majority of isolates derived after progression of disease are CXCR4-tropic. As well, it has been reported that viral isolates derived from transition of HIV-1-infected nonprogressors to progressors are dual-tropic (CCR5 and CXCR4).³⁵ It has also been demonstrated that CXCR4-tropic phenotype of recombinant HIV-1 strains and primary HIV-1 isolates are linked to a highly pathogenic phenotype in *ex vivo* lymphoid tissues, which is substantially inhibited by interfering with CXCR4-mediated entry.³⁶ It was then demonstrated that the specificity of recombinant HIV-1 strains for CXCR4-tropism correlated with potent depletion of CD4⁺ T-lymphocytes in human lymphoid histocultures.³⁷

Nevertheless, the development of moieties to inhibit or block interactions between the HIV-1 envelope and chemokine coreceptors is somewhat problematic. One of the problems in designing antichemokine coreceptor therapeutic agents is the complexity of the chemokine receptor–ligand interactions.³¹ This represents blocking of a tricomplex, gp120–CD4–CXCR4. The natural ligand of CXCR4 has been identified as stromal cell-derived cofactor (SDF-1), an alpha chemokine. SDF-1 α blocks the infection of T-lymphocytes with CXCR4-tropic strains of HIV-1.²⁷ Based on these data, molecular therapeutics in attempts to downregulate CXCR4 expression on the surface of relevant target cells have become an area of active investigation. As approximately 50% or more of individuals infected with HIV-1 convert from CCR5 to CXCR4-tropic viral strains during progression to the acquired immune deficiency syndrome (AIDS),³⁵ we hypothesized that CXCR4 would be a potentially important target for significant downmodulation utilizing newly developed RNAi technologies.

In this report, it is demonstrated that siRNAs specific to CXCR4 mRNA can downregulate this chemokine coreceptor's expression on the surface of target cells. As well, the siRNA negatively affects HIV-1 fusion with CXCR4-tropic viral envelopes.

Results

Suppression of CXCR4 and APJ expression on cell surfaces by siRNA

To analyze the ability of CXCR4 siRNA to suppress specifically CXCR4 expression, CXCR4-GFP (GFP: green fluorescent protein) or APJ-GFP expression vectors and siRNAs were cotransfected into HOSCD4 cells. As shown as Figure 1a, 48 h after transient transfection, GFP siRNAs efficiently decreased expression of both fusion proteins, CXCR4-GFP and APJ-GFP, while CXCR4 siRNA only reduced CXCR4-GFP expression. Cotransfection with APJ-GFP and CXCR4 siRNA had no effect on expression of APJ-GFP. A 2 nM concentration of siRNAs was used in these studies, as inhibition of CXCR4 expression dropped off sharply below this input concentration and no further clear decreases in CXCR4 levels were noted with increased levels of siRNA input. Of note, no cellular toxicity was noted in transfections using these siRNAs (not illustrated).

We then evaluated siRNA specificity on target gene expression in stable cell lines. The results illustrated in Figure 1b demonstrate that GFP siRNA efficiently downregulated APJ-GFP expression in HOS cells stably expressing CD4 and APJ-GFP, while CXCR4 siRNA had no effect on APJ-GFP expression in these cells, as assayed via immunofluorescence microscopy. Extended experiments were carried out to determine whether siRNAs suppress specifically CXCR4 and APJ expression, in stable 293 cell lines expressing CXCR4-GFP and APJ-GFP, respectively. The data in Figure 1c demonstrate that, in stably transfected 293 cell lines, CXCR4-GFP was dramatically downregulated by transfection of CXCR4 siRNA but not by APJ siRNA. Of note, conversely, APJ-GFP expression was suppressed only by APJ siRNA but not by CXCR4 siRNA. As such, these data suggest that GFP fusion proteins with relevant chemokine corecep-

Figure 1 Specificity of the effects of CXCR4 siRNA on target gene expression. The HOS CD4 cell line was cotransfected with constructs expressing APJ-GFP or CXCR4-GFP fusion proteins and 2 nM of CXCR4 siRNA or GFP siRNA (a). The stable cell line HOS CD4 expressing APJ-GFP (b) and the 293 cell lines expressing CXCR4-GFP or APJ-GFP (c) were transfected with 2 nM CXCR4 siRNA or APJ siRNA. The cells were fixed 48 h later and analyzed by fluorescence microscopy. The lower panels in (b) demonstrate cell numbers by 4',6'-diamidino-2-phenylindole hydrochloride (DAPI) staining of the nuclei (blue). The results shown are representative of at least two independent experiments.

tors can be downregulated by siRNAs, and thus function as a initial 'proof of concept' for this approach.

Downregulation of CXCR4 expression

A full series of HOS indicator cells were then transfected with siRNA specific to CXCR4 to quantify the siRNAs' potency in downregulating CXCR4. siRNA specific to GFP as a target was used as a negative control. As noted above, the specificity of GFP siRNA was confirmed previously in HOSCD4APJ-GFP cells (Figure 1a and b). We then sought to demonstrate the specificity of CXCR4 siRNA. As shown in Figure 2, in HOSCD4CXCR4 cells, CXCR4 was downregulated with at least 80% efficiency by CXCR4 siRNA, but only minimal decreases in CXCR4+ cells were noted with GFP siRNA, as analyzed by FACS. CCR5 expression revealed no change in the HOSCD4CCR5 indicator cells, with either siRNA moiety. Thus, these results provided evidence that CXCR4

siRNA specifically inhibits CXCR4 expression on cell surfaces, although full and absolute downregulation of CXCR4 was not obtained with transfected siRNAs.

Activities of CXCR4 siRNA in blocking CXCR4-mediated cell membrane fusion

We then utilized a well-characterized, luciferase-based cell-cell fusion assay to evaluate the abilities of CXCR4 siRNA in blocking CXCR4- and HIV-1 gp120-mediated cell membrane fusion. As effector cells, 293 cells were co-infected with vaccinia virus-encoded HIV-1 gp120 and vaccinia virus-encoded luciferase under the control of the T7 promoter. This particular target cell system was chosen as it lends itself to clear quantitation of HIV-1 envelope-induced cell fusion.³⁸ HOSCD4CXCR4 target cells were cotransfected with T7 RNA polymerase and CXCR4 siRNA. The results and relative percentages of changes in fusion activity are presented in Figure 3. Transfection of CXCR4 siRNA resulted in approximately 65 and 75% inhibition of CXCR4 coreceptor activity for both IIIB (CXCR4-tropic, Figure 3a; $P \leq 0.003$ by Student's *t*-test) and 89.6 (dual-tropic (CXCR4 and CCR5, Figure 3b; $P \leq 0.003$)) HIV-1 isolates, respectively. No blocking of CCR5 coreceptor activity was noted with the CXCR4 siRNA (Figure 3c; $P = 0.63$ (NS)), and GFP siRNA, as negative control, had no inhibitory effects on CXCR4-mediated cell fusion (Figure 3). As such, these data demonstrate a selective inhibitory effect on HIV-1-induced cell fusion by siRNA to CXCR4. *In vitro* fusion assays are known as usually difficult systems in which to demonstrate inhibitory effects with other anti-CXCR4 moieties.³⁹ Of note, though, in this system we reported previously that a specific CXCR4 inhibitor, ALX40-4C, inhibited over 80% of cell:cell fusion at micromolar concentrations.³⁸ These are interesting findings, as cell-to-cell fusion is likely a critical mechanism of HIV-1 spread within lymphoid tissues of infected individuals.

Inhibition of HIV-1 production by CXCR4 siRNA analyzed using cell-free viral infections

The effects of downregulated CXCR4 by siRNA were also evaluated using the cell-free HIV-1 T-tropic (CXCR4) strain NL4-3 and M-tropic (CCR5) strain ADA to infect the HOS cell-line series. No toxicity on cell proliferation was detected with these siRNAs (not illustrated). As illustrated in Figure 4a, inhibition of viral infection using a CXCR4-tropic virus (ie NL4-3) was demonstrated in CXCR4+ cells using the CXCR4 siRNA ($P \leq 0.03$), but the inhibition was relatively modest. The control siGFP had little effect on viral growth in these cells. Finally, the siRNAs did not alter R5-tropic viral growth on CCR5+ cells (Figure 4b; all *P*-values were not significant). Of interest, though, siRNA's effects on CXCR4 was more robust in inhibiting HIV-1 fusion (Figure 3), as compared to cell-free virion infectivity. This divergence in effects of cell-free virus infection *versus* cell:cell fusion, with efficient but not total downregulation of CXCR4, is important as it can be hypothesized to be secondary mechanistically to differential utilization of low levels of CXCR4 surface densities required for these viral processes.

To further analyze the effects of downregulation of CXCR4 by siRNA on cell-free viral infection, a complementary system was utilized in which luciferase-express-

Figure 2 Suppressed CXCR4 expression by CXCR4 siRNA. The HOS series of indicator cells (HOSCD4, HOSCD4/CXCR4 and HOSCD4/CCR5) were transfected or left untransfected with 2 nM of CXCR4 siRNA or GFP siRNA in each well of six-well plates, as indicated in the panels. Cells were harvested 48 h later and analyzed by fluorescence-activated cell sorting (FACS). The cells were stained by using mouse anti-human CXCR4 or CCR5 as an initial antibody (noted at the top of the figure), followed by PE-conjugated anti-mouse IgG as a secondary antibody. The results shown are representative of at least two independent experiments. SiX4, siRNA to CXCR4; siGFP, siRNA to GFP.

sing reporter viruses were produced and used to infect U87MG cells (astrocytic glial cell lines), expressing CD4 alone, CD4/CXCR4 or CD4/CCR5. As expected, no obvious HXB2-*luc* growth was detected on CD4/CCR5-expressing cells, but quite reasonable viral growth was demonstrated on CD4/CXCR4-expressing cells with a moderate decrease in cultures treated with CXCR4 siRNA (siCXCR4 versus siGFP- $P \leq 0.03$ (ANOVA – analysis of variance: F-test); siCXCR4 versus nontransfected – $P \leq 0.004$; siGFP versus nontransfected – $P = 0.11$ (NS)) (Figure 4c). These studies confirmed that CXCR4 siRNA could inhibit cell-free virus infection, as measured by luciferase activity, with X4-tropic reporter viruses, but less effectively than cell–cell fusion.

Discussion

This report demonstrates the ability of siRNAs to inhibit HIV-1-induced fusion, by targeting the mRNA for the

CXCR4 chemokine coreceptor. We used complementary approaches to demonstrate both CXCR4 surface down-regulation after treatment with specific siRNAs and their activities in altering HIV-1 fusion. Of note, the specificity of this effect was demonstrated by the lack of inhibition of dual-tropic viral isolates in CCR5+ and CD4+ target cells, and the lack of downregulation of chemokine coreceptors other than CXCR4. As well, siRNA targeted to another chemokine coreceptor (ie APJ) had no non-specific effects on CXCR4. Inhibition of cell-to-cell fusion via siRNA is especially promising, as it is difficult to efficiently demonstrate this effect using most chemokine coreceptor antagonists.^{39–42} Finally, inhibition of cell-free virion infection of CXCR4+ cells by CXCR4-tropic virus was demonstrated with the RNAi approach, without inhibition of CCR5-tropic viruses on CCR5+ cells, but the potency was clearly lower than for cell–cell fusion.

RNAi has promise as a potent molecular therapeutic approach in attacking the life cycle of a wide variety of

Figure 3 Effects of CXCR4 siRNA on cell–cell fusion mediated by CXCR4 and HIV-1 gp120. HOSCD4CXCR4 (a, b) and HOSCD4CCR5 (c) target cells were cotransfected with siRNAs and plasmids encoding luciferase under the control of the T7 promoter. 293 effector cells were infected with a vaccinia virus encoding T7 polymerase, and a vaccinia virus encoding strain-specific HIV-1 envelope proteins. Cell mixtures were maintained at 37°C for 4–6 h. Fusion was determined by a luciferase assay. The numbers below each figure represent effects of siRNA on fusion activity as a percentage of the positive controls. Negative controls lacking HIV-1 envelopes are usually below 5% in this assay system. The values are the means $\pm$ s.d. of quadruplicate samples, and the results are representative of two independent experiments.

human viral pathogens. Molecular therapy against HIV-1 has used both protein- and RNA-based modalities (for a review, see reference Dornburg and Pomrantz²⁴). Previous RNA-based modalities, which have had some

significant effects on HIV-1 replication in various model systems, include antisense, molecular decoys and ribozymes. The potential potency of RNAi may have importance as an antiviral agent, as compared to these other approaches. Previous studies have demonstrated siRNAs' inhibitory effects on polio virus, and recently human papilloma virus (HPV), hepatitis C virus (HCV) plus influenza.^{43–46}

Effects on both cellular cofactor RNAs and HIV-1-specific RNAs have also been demonstrated recently with siRNAs.^{47–52} An initial study has shown that CD4 mRNAs may be targeted by siRNAs as potential therapeutic interventions.⁵¹ One possible problem with this approach is the important detrimental immunomodulation of T-cell helper function, based on the robust depletion of the CD4 antigen on lymphoid cell surfaces. In addition, nuclear factor-kappa B (NF- κ B), which has been demonstrated to be critical in HIV-1 transcriptional control, has also been targeted with RNAi with some success (ie approximately 40–50% decrease in HIV-1 expression).⁵² A very recent study suggests that RNAi targeting CCR5 mRNA may be useful in inhibiting HIV-1 strains found relatively early in clinical disease (ie about 65% decrease in HIV-1 p24 antigen expression in these infectivity studies).⁵³ Finally, both structural protein (*gag*) and regulatory protein (eg *tat* and *rev*) viral mRNAs have also been approached using siRNA inhibitors in relevant model systems *in vitro*.^{47–50}

Of note, one report has demonstrated the potentially very important finding that siRNAs may gain entrance to the preintegration complex of HIV-1, leading to cleavage of the 'in-coming' viral genome.⁴⁹ In addition, this same study demonstrated that siRNAs could be produced from hairpin motifs produced from transfected plasmids. This allows the constant or regulated intracellular expression of siRNAs, which are cleaved from the hairpin motifs produced in the plasmid-transfected cells. Of note, transfected siRNAs although potent in many scenarios have a relatively short inhibitory time course on their target RNAs, unless *in situ* amplification occurs with this process.⁴⁷ As noted previously, it is not yet clear that RNAi amplification is a productive process in mammalian cells. As well, whether the RNAi effect spreads potentially enough between cells to inhibit HIV-1 at a distance from the initial site of RNAi is not clear.

The CXCR4 coreceptor appears to be an important target for therapeutic intervention against HIV-1. A variety of antibodies, small molecular inhibitors and intracellular single-chain variable fragments (SFVs) have been used both *in vitro*, and some *in vivo*, to inhibit CXCR4-specific binding of HIV-1 in relevant target cells.^{39–42,54–60} Of note, the *in vivo*-tested molecular inhibitors of CXCR4 did not appear to lead to significant adverse immunological effects.⁴² In addition, targeting cellular proteins that act as cofactors in the HIV-1 life cycle may be of greater reliability than targeting viral mRNA species or genomic RNA with RNAi, as the propensity of this virus to undergo rapid mutations would alter the long-term inhibitory capabilities of siRNAs directed against the virus itself.

In the present study, we have also developed an siRNA to the orphan G-coupled seven transmembrane receptor APJ. This unique coreceptor has been shown to bind and allow infection of cells by a number of HIV-1 strains.^{61–64} Its presence in the central nervous system

(CNS) is of potential importance,^{64–66} and investigation of siRNAs' effects on this moiety is ongoing in our laboratories. CXCR4, as well, has been demonstrated on CNS-based cells.^{66,67} It has been recently demonstrated that RNAi also functions in mammalian neurons *in vitro*.⁶⁸ As such, targeting CXCR4 and/or APJ in human CNS cells may be an important first step in targeting RNAi toward HIV-1-induced encephalopathy.

During preparation of this manuscript, an initial report suggested that siRNAs could be useful in

inhibiting both CCR5 and CXCR4 for intervening in HIV-1 infection.⁶⁹ This relatively small study suggested that only a 60% decrease in CXCR4+ cells by siRNA led to very significant decreases in viral replication. It was surprising in this report that the level of viral production after only 48 h postinfection was above 150 ng/ml of p24 antigen equivalents in U87 indicator cells, even with zidovudine added after initial infection to prevent a second round of viral spread in these experiments. In addition, our present study is the first to demonstrate that siRNAs against CXCR4 can potently inhibit HIV-1-mediated cell-to-cell fusion, a critical parameter altered by siRNA inhibition of CXCR4 activity, important for both intra- and interhost HIV-1 transmission *in vivo*, and not evaluated in the report by Martinez *et al*.

In our present study, inhibition of fusion by siRNA to CXCR4 was actually more robust than inhibition of cell-free virion infectivity, with the approximately 80% downregulation of CXCR4 that we achieved with this siRNA moiety. This is mechanistically interesting and may be secondary to differences in low chemokine coreceptor levels and densities on target cell surfaces required for these two processes. Of importance and paradoxically, previous studies of pharmacological drugs or small molecule inhibitors that target HIV-1 entry have shown them to be dramatically more potent at inhibiting cell-free viruses, as compared to cell:cell fusion.⁷⁰ Of note, inhibition of CCR5-directed HIV-1 cell-to-cell fusion by siRNAs was also not analyzed in a recent study,⁵³ as only effects on infection with cell-free viruses were reported and their results were comparable to our findings. Although, in the present study, full downregulation of CXCR4 was not demonstrated using transfection approaches of siRNAs, certain modifications are possible in further model systems to increase efficiency. Future studies in primary T-lymphocytes of RNAi directed against CXCR4 will likely require transduction of these cells with retroviral vectors expressing hairpin motifs, which produce specific siRNAs within the cells.⁴⁹ Inducible promoters will likely also be quite useful in these approaches.

In conclusion, these present studies demonstrate that RNAi can inhibit the expression of the critical HIV-1 chemokine coreceptor CXCR4. The utility of this approach toward inhibiting HIV-1 in peripheral blood and lymphoid tissue, as well as in possible reservoir or sanctuary sites such as the CNS, requires further investigation. Thus, the potential potency and utility of the RNAi approach to target multiple sites in the HIV-1

Figure 4 Inhibition of cell-free HIV-1 infection of CXCR4+ cells. The HOS cells, CD4/CXCR4 and CD4/CCR5, were used as targets for HIV-1 infection, 48 h after transfections with siRNAs targeting CXCR4 or GFP mRNAs. A 10 ng portion of HIV-1 p24 antigen equivalents of viral strains (a) NL₄₋₃ and (b) ADA was utilized. After vigorous washing, viral outgrowth was assayed 72 h later, utilizing ELISA for HIV-1 p24 antigen expression. These data are from duplicate cultures with standard error bars, and are representative of two independent studies. (c) HIV-1 luciferase reporter virus infections. U87MG cells, expressing CD4 alone, CD4/CXCR4 or CD4/CCR5, were used as targets for HIV-1_{HXB2} luciferase reporter virus, 48 h after siRNA transfections (2 nM). The cells, lysed 24 h after infection, were analyzed for luciferase activity (RLU/s) in a luminometer. As expected, the HIV-1 ADA luciferase reporter virus did not grow to significant levels on CD4/CXCR4+ cells (not illustrated). The data illustrated are from triplicate cultures with standard error bars, and are representative of two independent experiments.

life cycle may allow clinically 'intracellular immunization' against HIV-1 in certain infected individuals. Clearly, though, solving technical difficulties in the use of siRNAs to treat HIV-1-infected patients is also predicated on a suitable and efficient *in vivo* delivery system.

Materials and methods

Materials

Synthetic siRNA duplexes were designed and then chemically synthesized by Dharmacon Inc. (Lafayette, CO, USA). The 21-nucleotide RNA duplexes had two-nucleotide deoxythymidine 3' overhangs. The sequences of siRNA were as follows: CXCR4, 5'-GCA UGA CGG ACA AGU ACA G dTdT-3' (sense), 5'-CUG UAC UUG UCC GUC AUG C dTdT-3' (antisense); GFP 5'-GCA

GCA CGA CUU CUU CAA G dTdT-3' (sense), 5'-CUU GAA GAA GUC GUG CUG C dTdT-3' (antisense); APJ, 5'-GGU GCA GUG CUA CAU GGA C dTdT-3' (sense), 5'-GUC CAU GUA GCA CUG CAC C dTdT-3' (antisense). See Figure 5 for illustration of these siRNAs located on the homologous regions of the CXCR4, GFP and APJ mRNAs.⁶⁴ Lipofectamine 2000 reagent was purchased from Invitrogen Inc. (Carlsbad, CA, USA) and Fugene 6 was purchased from Roche Inc. The full open reading frames of CXCR4 and APJ were amplified using PCR and subcloned in-frame into *Hind*III and *Bam*HI sites of the pEGFP-N1 vector, to create GFP fusion constructs.⁶⁴ All constructs were sequenced to confirm the correct sequence and orientation.

Cell cultures and transfections

HEK293 cells and the indicator human osteosarcoma cells, HOSCD4CXCR4 and HOSCD4CCR5 (AIDS Reagent

Figure 5 Location of siRNAs on homologous regions of CXCR4, APJ and GFP mRNAs. This schematic diagram illustrates the nucleotide homologies of the double-stranded siRNAs used in these studies for CXCR4, APJ and GFP. Genbank accession numbers for these target mRNAs are included. The sense strand is red and the antisense strand is blue in this diagram. Of note, 3' dTdT overhangs are included on both strands.

Repository, NIH), were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) with 0.5 µg/ml puromycin. HOSCD4CXCR4-GFP, HOSCD4APJ-GFP, 293-APJ-GFP and 293-CXCR4-GFP stable cell lines have been described previously.^{31,64} Cells were transfected at 90% confluence in six-well plates with plasmid DNA and siRNA (2 nM) by using Lipofectamine 2000 or Eugene 6, according to the manufacturer's instructions. Cells were split after 1 day into Nunc two-well chamber slides for observation with fluorescence microscopy and in 24-well plates for HIV-1 infection assay. Fluorescence microscopy was performed on an Olympus System microscope, model BX60, with fluorescence attachment BX-FLA.⁶⁶

Fluorescence-activated cell sorting (FACS)

siRNA-transfected cells and control cells were harvested 48 h post-transfection by washing twice with phosphate-buffered saline (PBS: Ca²⁺ and Mg²⁺ free) and detaching with 1 mM EDTA containing PBS. The cells were further washed with PBS, and treated with initial and secondary antibodies (1:100 antibody in 3% BSA/PBS) with incubation at 4°C for 40 min, sequentially. Antibodies used in flow cytometry were as follows: anti-human CXCR4 (Cat#36190D), anti-human CCR5 (Cat#36461A) and PE anti-mouse Igκ (Cat#559940) purchased from Pharmingen Inc. (San Diego, CA, USA). The cells were then analyzed in the flow cytometry core facility at Thomas Jefferson University.

Gene reporter fusion assay

A well-characterized gene reporter fusion assay was used to determine the coreceptor activity of CXCR4 in mediating HIV-1 viral entry, following a modified procedure published previously.^{70,71} Briefly, the effector 293 cells were infected with recombinant vaccinia virus expressing HIV-1 Env proteins from several viral strains and T7 RNA polymerase for 2 h. Infected cells were then trypsinized, washed with PBS, resuspended in medium and incubated overnight at 32°C in the presence of rifampicin (100 mg/ml). HOSCD4CXCR4 cells as targets were cotransfected in six-well plates with plasmids encoding luciferase under control of the T7 promoter and CXCR4 siRNA, using the Lipofectamine 2000 reagent. At 4 h after transfection, cells were lifted, seeded in 24-well plates and incubated at 37°C overnight. To initiate fusion, 10⁵ effector cells were added to each well and incubated at 37°C. After 4 h of fusion, cells were lysed in 150 µl of reporter lysis buffer (Pharmingen) and assayed for luciferase activity by using commercially available reagents (Pharmingen), with an FB12 Lumino-meter (Zylux Corporation, Maryville, TN, USA).

HIV-1 infection assays

To further determine anti-HIV-1 activity of siRNA against the coreceptor CXCR4, HOSCD4, HOSCD4CXCR4 and HOSCD4CCR5 cells (near-confluent cultures in six-well plates) were transfected with siRNA against CXCR4, and GFP as a control. At 2 days after transfection, the cells were infected with 10 ng of p24 antigen equivalents of each virus strain overnight, and then thoroughly washed three times. The CXCR4-tropic virus, NL₄₋₃, and the CCR5-tropic virus, ADA, were used in these studies. The supernatants were collected 3 days after infection for

p24 antigen measurements using enzyme-linked immunosorbent assays (ELISA, Zeptometrix).⁴⁰

HIV-1 proviral clones, expressing the luciferase (*luc*) gene in the *nef* open reading frame of HXB2 (X4-tropic) and ADA (R5-tropic) backbones,⁴⁸ were used to produce viral stocks after transfection into 293 T cells. Normalized quantities of virus were used to infect U87-MG astrocytic-glia cells constitutively expressing CD4 alone, CD4 and CXCR4, or CD4 and CCR5 on their surfaces, 48 h after transfection of siRNA (2 nM). These cell lines were propagated in DMEM with 10% FBS and 1 µg/ml puromycin. At 24 h after viral infection, cells (2–8 × 10⁵) were lysed in reporter lysis buffer and assayed for luciferase activity, as per the manufacturer's instructions (Pharmingen), in a luminometer.³¹

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