Efficient Gene Transfer of HIV-1-Specific Short Hairpin RNA into Human Lymphocytic Cells Using Recombinant Adeno-associated Virus Vectors

Daniel Boden,* Oliver Pusch,* Fred Lee, Lynne Tucker, and Bharat Ramratnam

Laboratory of Retrovirology, Division of Infectious Diseases, Department of Medicine, Brown Medical School, Providence, RI 02903, USA

*These authors contributed equally to this article.

The cellular introduction of short, interfering RNA leads to sequence-specific degradation of homologous mRNA, a process termed RNA interference (RNAi). Here, we report that recombinant adeno-associated virus 2 (rAAV-2) can be used to transfer short hairpin (sh) RNA expression cassettes genetically into human cells. HIV-1 replication was suppressed by >95% in H9 cells and primary human lymphocytes that expressed shRNA targeting the first exon of the viral transactivator protein tat compared to control cells. rAAV-2 integrated stably into the host genome, leading to long-term expression of tat shRNA. Our findings demonstrate the utility of rAAV-2 for the genetic transfer of shRNA expression cassettes into human cells, providing an alternative to using retroviral vectors as RNAi delivery systems.

Key Words: adeno-associated virus, HIV-1 gene therapy, RNA interference

INTRODUCTION

RNA interference (RNAi)-mediated silencing of human immunodeficiency virus type 1 (HIV-1) replication can be achieved by transfection of short interfering RNA (siRNA) or DNA vectors containing siRNA expression cassettes. Recent work has demonstrated that cellular expression of siRNA targeting HIV-1 proteins such as tat, rev, and gag inhibits virus replication, raising the possibility of using RNAi as a genetic therapy for chronic HIV-1 infection [1–7]. Application of antiviral RNAi in a clinical setting will require the use of efficient vector delivery systems capable of mediating long-term, stable siRNA expression.

Recombinant adeno-associated virus (rAAV) is an attractive vehicle for gene therapy that offers several advantages over other vector systems of viral origin. A major safety concern of retroviral vectors is the insertional activation of cellular proto-oncogenes resulting from random integration of provirus into the host genome. One of the most attractive features of AAV is its ability to integrate site-specifically into the AAVS1 region of chromosome 19, thereby reducing the mutagenic and carcinogenic potential of somatic gene transfer [8–11].

Due to the requirement of helper virus for productive infection, adenovirus-associated viruses are naturally replication-deficient paroviruses and have not been associated with any human disease [12]. Moreover, AAV-2 exhibits a broad host range and infects a wide variety of tissues and cells [13–17]. AAV-2-derived viral vectors have been used successfully for efficient long-term gene expression in both dividing and nondividing cells [13–16]. As opposed to retroviral vectors, recombinant AAV vectors are devoid of endogenous promoters, thereby excluding the possibility of promoter interference between an upstream viral promoter (e.g., LTR) and an internal promoter of an expressed transgene [17,18].

Given previous demonstration of AAV-mediated delivery of therapeutic RNA molecules such as ribozymes [19,20], we sought to determine the utility of AAV-2 for the stable genetic transfer of short hairpin RNA (shRNA) expression cassettes into a lymphocytic cell line and primary human lymphocytes. Employing this system, we tested the antiviral activity of shRNA that targets the HIV-1 transactivator protein tat.
RESULTS AND DISCUSSION

Antiviral Activity of a tat shRNA Expression Construct

We introduced the shRNA expression cassette into a recombinant AAV DNA vector. The cassette consisted of an upstream MTD (modified-tRNA-derived) Pol III-type promoter followed by a short hairpin DNA sense and antisense sequence, separated by a hexaloop (Fig. 1). We chose the MTD promoter given the recent demonstration that it more effectively mediates HIV-1-specific RNAi compared to other Pol III promoters such as U6, U6+1, and H1 [21]. We determined the tat shRNA target sequence by alignments of computer-predicted secondary structures (mfold 2.0) of HIV-1 tat exon 1. We selected the five lowest free energy structures for the analysis and scanned 21- to 23-nt sequence stretches that were conserved among all energy structures for potential siRNA target sites. The resulting construct pAAV-tat contained a 22-nt target sequence with favorable predicted secondary structure features such as free energy ($\Delta G$) of the target site, total numbers of unpaired nucleotides, and major loop size. We constructed a control vector (pAAV-luc) by introducing luciferase-specific sequences into the shRNA expression construct.

Antisense sequence, separated by a hexaloop. A five-thymidine transcription termination signal was placed downstream of the hairpin sequence. To allow the generation of shRNA-expressing cell lines, a SV40 early promoter-driven neomycin resistance gene was introduced downstream of the hairpin expression cassette. The neomycin cassette was replaced with a GFP reporter cassette to determine infectious titer of rAAV-2 by flow cytometry.

FIG. 1. Schematic representation of a short hairpin RNA expression cassette introduced between the inverted terminal repeats (ITRs) of a recombinant AAV-2 DNA vector. The MTD promoter was used to drive expression of a tat/luc hairpin transcript consisting of a 21-nt sense and antisense sequence separated by a hexaloop. A five-thymidine transcription termination signal was placed downstream of the hairpin sequence. To allow the generation of shRNA-expressing cell lines, a SV40 early promoter-driven neomycin resistance gene was introduced downstream of the hairpin expression cassette. The neomycin cassette was replaced with a GFP reporter cassette to determine infectious titer of rAAV-2 by flow cytometry.

downstream of a Pol II promoter-driven neomycin resistance gene or GFP reporter gene. Infectious particles were generated after cotransfection of 293T cells with equimolar amounts of AAV cloning, packaging, and helper vectors. We chose H9 cells, which are highly permissive for HIV-1 infection, for the AAV transduction. The cells were preincubated with DNA synthesis inhibitors based on previous reports that AAV transduction of cells is enhanced by chemical induction using certain DNA-damaging agents [22]. We determined the transduction efficiency of this method using rAAV-hrGFP and it revealed consistent transduction efficiencies of about 30%, determined by flow cytometry for GFP expression. Finally, we transduced H9 cells with rAAV-tat and rAAV-luc, which contain in addition to the shRNA expression cassette the neomycin drug resistance gene, and placed them under drug selection for 4 weeks.

Verification of Genomic Integration of rAAV

Previous work has revealed that the most common integration site of AAV into the host genome is at AAVS1, which is located on human chromosome 19 within 19q13.4 [8,23,24]. To verify rAAV-2 genomic integration, we performed an AAVS1-specific AAV PCR as previously described by Huser et al. [10] and verified the results by Southern blot analysis. We subjected DNA extracted from rAAV-2-transduced and neomycin-selected H9 cells to a two-step AAVS1 AAV PCR (Fig. 2A). The first-step PCR includes the pAAV-tat-specific forward primer PAV-s and the reverse primer PAAVS1-as, which is complementary to the region in the AAVS1 gene of chromosome 19. The second-step PCR consists of two AAV-specific primers. As shown in Fig. 2B, integration of recombinant AAV into the AAVS1 gene was demonstrated in the genomic DNA of stably transduced H9 cells compared to the appropriate controls. We included, minus-first-step PCR sample (Fig. 2B, lane 5) to rule out contamination and/or coamplification of episomal rAAV in the second-step PCR.

To verify these findings, we performed a Southern blot on DNA isolated from rAAV-2-transduced and neomycin-selected H9 cells. The Southern blot shows genomic integration of recombinant AAV-2 (Fig. 2C). The two bands observed in the EcoRV lane may indicate two different integration events within AAVS1. It is known that AAV can integrate in various positions in the 4-kb-long AAVS1 site [10]. One EcoRV site is present within the AAVS1 site and two additional EcoRV sites are located approximately 5.4 kb upstream and 5.8 kb downstream of AAVS1. It is conceivable that one integration event leaves the internal AAVS1 EcoRV site intact, while the other integration event removes the EcoRV site. AAV integration events can lead to disruption, deletions, and rearrangements of the AAVS1 target [25,26] with chromosomal deletions up to 2 kb [26]. Thus, the potential removal of the EcoRV site within AAVS1 combined with different degrees of chromosomal deletions may
explain the observed two bands indicating two separate integration events.

Quantification of \textit{tat} shRNA Expression in Stably Transduced Cell Lines

We quantified precursor \textit{tat} shRNA expression levels in transduced H9 cells by real-time PCR over a 21-day period. The MTD promoter belongs to the class II polymerase III promoters that are intragenic and cotranscribed with the \textit{tat} shRNA. The total length of the resulting transcript including the shRNA is 150 bp and can therefore be readily detected by RT-PCR. We subjected total RNA to reverse transcription and real-time PCR. Input RNA amount was normalized by GAPDH PCR. Fig. 3 shows the expression of MTD \textit{tat} shRNA transcripts in transduced and neomycin-selected cells at different time points after HIV-1 infection. The results demonstrate stable expression of \textit{tat}-specific shRNA transcripts up to day 21 of viral infection, corresponding to day 48 posttransduction with rAAV-\textit{tat}.

HIV-1 Challenge of Transduced H9 Cells and Primary Lymphocytes

We infected H9 cells permanently expressing \textit{tat} or \textit{luc} shRNA with 100 TCID$_{50}$ of HIV-1NL4-3. We assayed culture supernatant for p24 antigen production over a 3-week period. On day 21 HIV-1 replication was suppressed by 1200-fold in cultures of H9 cells expressing \textit{tat} shRNA, compared to cultures of cells expressing \textit{luc} shRNA (Fig. 4A). Northern blot analysis of total intracellular HIV-1 RNA confirmed the p24 antigen results with specific degradation of full-length HIV-1 RNA in infected H9...
cells expressing tat shRNA compared to control cells expressing luc shRNA on day 21 postinfection (Fig. 4B).

Next, we assessed whether AAV could be used to transfer HIV-1-specific RNAi to primary lymphocytes. We obtained peripheral blood mononuclear cells of an anonymous HIV-1 seronegative donor from the Rhode Island Blood Bank and depleted them of CD8+ T lymphocytes using magnetic beads (Dynal, NY, USA). We transduced the resulting CD4+ T lymphocyte-enriched population with AAV-tat and AAV-luc. For these experiments, the neomycin resistance gene was replaced with blasticidin to allow for rapid selection of transduced cells. After 1 week of drug selection in blasticidin (1 μg/ml), we placed the CD4+ T lymphocytes in medium containing IL-2 (10 U/ml) and PHA (2 μg/ml) and challenged them with 1000 TCID50 (50% tissue culture infectious dose) of HIV-1NL4-3. Measurement of p24 antigen level in culture supernatant 3 days after viral challenge revealed a 21-fold reduction of HIV-1 replication in CD4+ T lymphocytes expressing tat shRNA compared to cells expressing luc shRNA. This degree of inhibition is comparable to that of recent studies employing lentiviral delivery of si/shRNA expression cassettes targeting both HIV-1 gene products and accessory cellular proteins required for the viral life cycle (e.g., the CCR5 coreceptor) [27,28].

RNA interference offers a powerful technique to inhibit selectively the expression of disease-related genes such as oncogenes [29]. RNAi can inhibit the replication of viruses such as HIV-1, hepatitis C, and human papillomavirus [30–32]. Thus far, permanent expression of siRNA has been most frequently achieved by the use of retroviral delivery systems [33]. Recent work has demonstrated that lentiviral constructs are able to engineer RNAi in primary lymphocytes and stem cells [27,34]. Although these systems allow successful transduction of a wide variety of cell types, their application to human diseases may be limited given the possibility of insertional activation of cellular oncogenes by random integration of retrovirus into the host genome, as recently observed in a gene therapy trial for human severe combined immunodeficiency-X1 disease [35,36].

Our results demonstrate the utility of rAAV-2 in achieving stable expression of HIV-1-specific shRNA in human lymphocytic cells and primary lymphocytes. Despite the availability of highly active antiretroviral therapy, the dynamic replication kinetics of HIV-1 ensures the eventual selection of viral species that escape drug and immune control. Nearly 50% of individuals who initiate highly active antiretroviral therapy will eventually fail treatment due to the emergence of drug-resistant virus. Currently, individuals who harbor multidrug-resistant HIV-1 have few therapeutic options. In the absence of new, effective antiviral agents, these patients will eventually progress to severe immunodeficiency and death. Control of HIV-1 replication by genetic transfer of HIV-1-specific shRNA to stem cells or lymphocytes ex vivo may be an option for these individuals to delay progression to AIDS. It is not yet known whether HIV-1-specific RNAi...
can durably suppress virus replication in a clinical setting. It is likely that the 20-fold inhibitory effect we observed in primary lymphocytes expressing tat shRNA in vitro will be insufficient to contain HIV-1 replication in vivo. Thus, an important research goal must be to devise more potent HIV-1-specific RNAi constructs. Several aspects of the RNAi machinery could potentially be manipulated to increase the efficiency of the resulting gene silencing. For example, it has been demonstrated that the choice of Pol III promoter impacts directly the potency of HIV-1-specific RNAi [21]. Alternatively, it may be possible to design vectors capable of delivering multiple shRNAs simultaneously and thus achieve a synergistic or additive effect. Further investigations are warranted regarding the optimization of HIV-1 RNAi and the clinical potential of rAAV-mediated genetic transfer of si/shRNA expression cassettes into human cells.

**Methods**

**Construction of MTD promoter-driven shRNA expression cassette.** The MTD promoter was generated by recombinational PCR using the following primers: TS, 5'-TGCTGGGCGCCATTAACCCAGGAAGTGCGATGATCG-3'; and 3'-AGACCCGCCTCTGTAATGCGCTTCTCGAGAGCTGGGTCTGTTTCGATCCATCGACCTCTGGGTTATGGGCCCAGCA-3'; Not-Ts, 5'-GGCGGGCGGCCAACAACTGAGTGCGCGACGGCGGGACGGAAGGTTGCAGTTCACGCTAGCATCAGGACACACGCGACTTCGTAAGGATCCGCTCAGAGGACATCAC-3'; and Not-Ts, 5'-GGCGGGCGCTGATGAGATGACCTAAACAGACAGATGTCGTCCGTCGAGTGTTAGC-3'. The resulting PCR product was cloned into the NotI site of the shuttle vector pCMV-MCS (Stratagene, Valencia, CA, USA), yielding the construct pCMV-MTD-MCS. For the production of stable cell lines, a shuttle vector pCMV-MCS (Stratagene, Valencia, CA, USA), yielding the producer cell line and transducing viral titers, humanized Renilla reniformis green fluorescent protein was introduced into the above restriction sites. The resulting shRNA expression cassettes were subcloned into the NotI site of pAAV-MCS (Stratagene). To incorporate the target shRNA sequence, two complementary DNA oligos were synthesized, annealed, and inserted between the Xhol and XhoI sites within the MTD promoter of pAAV-MTD-Neo. The DNA oligo sequences consisted of 21-mer sense and reverse complementary sequences separated by a 6-nt loop and Not-RAS, 5'-TCCGAGGACATCAC-3'. The resulting PCR product was cloned into the NotI site of the shuttle vector pCMV-MCS (Stratagene, Valencia, CA, USA), yielding the construct pCMV-MTD-MCS. For the production of stable cell lines, a shuttle vector pCMV-MCS (Stratagene, Valencia, CA, USA), yielding the producer cell line and transducing viral titers, humanized Renilla reniformis green fluorescent protein was introduced into the above restriction sites. The resulting shRNA expression cassettes were subcloned into the NotI site of pAAV-MCS (Stratagene). 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Quantiﬁcation of precursor shRNA expression level. Precursor tat shRNA expression levels in transduced H9 cells were assayed by real-time PCR over a 21-day period. Total cellular RNA was isolated by Trizol (Invitrogen) extraction and treated with RQ1 Dnase (Promega, Madison, WI, USA) according to the manufacturer’s protocol. One microgram of Dnase I-treated RNA was used for the RT reaction using PowerScript Reverse Transcriptase (Clontech, Palo Alto, CA, USA) following the supplier’s recommended usage. One microliter of cDNA was added to the PCR mix including 1× Titanq PCR buffer (Clontech); 20 pmol of sense primer MT-S, 5′-GGCAAGACCTGCAAGGTT-3′, and antisense primer MT-AS, 5′-CAAATTTGTCACAGCTTCATAG-3′; 1 mM dNTPs; SYBR Green I (1.75 μM); 10 mM Tris, and 1× Titanq polymerase (Clontech). RNA normalization was performed by GAPDH PCR with the primers G1, 5′-GAATTTCCTCCCTCCTGCTCATG-3′, and G2, 5′- CCCTGGCTGGGGTTGCTCAA-3′. Real-time PCR was carried out in an iCycler (Bio-Rad) using the following thermal cycling proﬁle: 95°C for 1 min and 35 × 95°C for 15 s, 61°C for 30 s, 68°C for 30 s.

HIV-1 infection of transduced target cells. Two million 293T cells were plated on 10-cm dishes in DMEM supplemented with 10% FBS. After 24 h 10 μg of the molecular infectious clone HIV-1NL4.3 was transfected into 50 million 293T cells using Lipofectamine 2000. Forty-eight hours later viral supernatant was harvested, spun down to remove any remaining cells, and ﬁltered through a 0.45-μm ﬁlter. Cells were infected with 1 ml of viral supernatant for 2 h at 37°C. After 2 h incubation, viral supernatant was harvested, spun down to remove any remaining cells, and ﬁltered through a 0.45-μm ﬁlter. Cells were infected with 1 ml of viral supernatant for 2 h at 37°C. Cells were washed twice in PBS, resuspended in 10 ml complete RPMI, and maintained in culture for 14 days. Cultures were harvested on day 14 postinfection, spun down, ﬁltered through a 0.45-μm ﬁlter, and stored frozen at −70°C for later use. The resulting viral stocks were titered on PHA-stimulated PBMCs and H9 cells and the TCID50 was determined on PHA-stimulated PBMCs and H9 cells and the TCID50 was determined by the method of Reed and Muench. Five million H9 cells transduced with MTD-luc and MTD-tat, respectively, were infected with 100 TCID50 of viral stock for 2 h at 37°C, washed twice with PBS, and resuspended in 5 ml CRPMI (10% FBS, penicillin 100 μU/ml, streptomycin 100 μg/ml) supplemented with 600 μg/ml neomycin. Five hundred thousand CD8+–depleted and PHA-stimulated peripheral blood lymphocytes transduced with MTD-luc or MTD-tat were infected with 1000 TCID50 of viral stock as described above for H9 cells. Cells were resuspended in CRPMI containing IL-2 (10 U/ml), PHA (2 μg/ml), and blastidin (1 μg/ml).

Northern blot for intracellular HIV-1 RNA. Total cellular RNA was extracted from RAV-tat-luc-transduced H9 cells 21 days after infection with HIV-1NL4.3. Ten micrograms of total cellular RNA was incubated in glyoxal sample loading dye (Ambion) for 1 h at 50°C, separated on a 1.2% agarose gel, and transferred to a nylon membrane using the Turbo blotter system (Schleicher & Schuell, Dassel, Germany). RNA was blotted on a Zeta-Probe GT membrane (Bio-Rad) and immobilized by UV crosslinking. HIV-1 RNA was detected with a 32P-random-labeled DNA probe (HIV-1NL4.3 position 1631–2130). Hybridizations were carried out at 65°C using UltraHyb-Oligo hybridization buffer (Ambion). The membranes were washed twice in 2× SSC, 0.1% SDS and 0.2× SSC, 0.1% SDS at 60°C. As an internal standard, a 500-bp 32P-random-labeled GAPDH DNA probe was used. Filters were stripped between hybridizations by incubation in 0.1× SSC, 0.7% SDS at 95°C.

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