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Improved Inhibition of Human Immunodeficiency Virus Type 1 Replication by Intracellular Co-overexpression of TAR and RRE Decoys in Tandem Array

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Intracellular expression of RNA decoys, such asTAR or RRE decoy, has been previously shown to protect immune cells from human immunodeficiency virus type 1 (HIV-1) replication by inhibiting the binding of the HIV-1 regulatory protein to the authentic HIV RNA sequence. However, HIV-1 challenge experiments of primary human T cells, which express the RNA decoy, demonstrated that the cells were only transiently protected, and hence, more improved protocols for HIV-1 inhibition with the RNA decoys need to be developed. In this report, in order to develop a more effective RNA decoy, we analyzed and compared the ability of a series of RNA decoy derivatives in inhibiting HIV-1 replication in CEM cells. Using an improved tRNA cassette to express high levels of RNA decoy transcripts in cells, we found that co-expression of both TAR and RRE decoys, in the form of an aligned sequence in a single transcription cassette, much more potently blocked cells from HIV-1 than the expression of only one kind of RNA decoy. This observation will have an important implication for experiments involving optimization of clinical applications in RNA decoy-based gene therapy against HIV-1.

***Key words*:** gene therapy, HIV-1, RNA decoy, RRE, TAR

Currently used anti-HIV-1 agents have marked limitations such as side effects, complicated dosing schedules, or occurrence of multidrug-resistant variants in many clinical circumstances (Martinez-Picado *et al*., 2000). Gene therapy would be a potential alternative and powerful approach for the specific modulation of HIV-1 infection. The reason is that the precursor of HIV-1 target cells, hematopoietic cells, can be *ex vivo* accessible to genetic manipulation without adverse consequences (Statham and Morgan, 1999). A number of strategies based on the intracellular expression of inhibitor genes have been developed for HIV-1 inhibition. Broadly, one can distinguish between protein-based inhibitors, such as transdominant mutant viral proteins or intracellular antibodies against viral proteins, and RNA-based inhibitors that include antisense RNA, ribozyme, RNA decoys, or siRNA (Gilboa and Smith, 1994; Sullenger and Gilboa, 2002). RNA-based inhibitors have more potential advantages because they are unlikely to be immunogenic, more easily expressed at high levels, and may be more specific, and therefore, are less likely to interfere with normal cellular functions. However, the use of antisense RNA, ribozyme or siRNA could be potentially limited because of the propensity of HIV-1 to generate escape mutants which will be resistant to the inhibitors.

RNA decoys are short oligonucleotides corresponding to important regulatory sequences on HIV-1 RNA termed the trans activation response (TAR) sequence or the Rev response element (RRE) sequence. The binding of HIV-1 encoded regulatory proteins, Tat and Rev, to TAR and RRE, respectively, is critical for the activation of HIV gene expression and, consequently, viral replication (Rosen and Pavlakis, 1990). We, as well as other groups, have previously shown that TAR and RRE decoys appear to inhibit HIV-1 expression and replication in the human CD4+ T cell line CEM by competing against authentic viral TAR or RRE sequences for the binding to Tat or Rev (Sullenger *et al*., 1990; Lee *et al*., 1994; 1995; 1998; Smith *et al*., 1996). A potential advantage of RNA decoys is that the generation of variant strains is less likely, relative to other RNA based inhibitors. This is because the RNA decoy target sequences are highly conserved, and an alteration in Tat or Rev, which will allow disruption in the binding process to the RNA decoy, would also impede binding to their authentic HIV-1 RNA sequences. However, attempts to inhibit HIV-1 replication in cultures of CD4+ T cell, taken from human peripheral blood, using these RNA decoys have been limited by low and transient level of protection of the cells from the virus (unpublished data). Thus, it will be necessary to improve the potency of

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the RNA decoys much more before application to the clinical anti-HIV-1 gene therapy protocol.

In this study, I have developed very effective RNA decoys by analyzing and comparing the activity of various RNA decoy derivatives that would protect CEM cells from HIV-1. Using a modified tRNA expression system to express high levels of RNA decoy derivatives in cells, we found that an aligned transcript of TAR and minimal RRE RNAs expressed in a single transcription unit was more potent than any single RNA decoy.

Materials and Methods

Cells and viruses

The HIV-1 virus strain used in this study was the ARV-2 isolate, which was propagated in HUT78 cells (Sanchez-Pescador *et al*., 1985) and supplied by Drs. Cheng and Levy. The CEM cell, which was provided by Dr. Nara, was a CD4+ human T-lymphocyte cell line which is highly susceptible to infection with HIV-1, including the ARV-2 isolate (Nara and Fischinger, 1988). CEM cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (GIBCO, USA).

Construction of retroviral vectors expressing RNA decoy and transduction of CEM cells

Oligonucleotides TAR, mRRE, TAR+mRRE, and TAR-L-mRRE were cloned between the *Sac*II and *Bam*HI site of the modified DCT vector previously developed (Lee *et al*., 1994; 1995). This modified vector contains the $tRNA_i^{met}$ gene (△3-2) which retains an functional 3′ RNA processing signal and also retains two stem loop structures (hpI and hpII) between $tRNA_i^{met}$ gene and transcription termination signal. In addition, these two stem loops were separated by short spacer sequences, L. The oligonucleotides representing various forms of RNA decoy sequences were inserted in the middle of the L sequence to generate DCT-TAR, DCT-mRRE, DCT-TAR+mRRE, and DCT-TAR-L-mRRE, respectively. Therefore, in these vectors, the oligonucleotide L was split into LI and LII. Vector DNA was converted to the corresponding virus as previously described (Lee, 1998). Briefly, 1 μg of plasmid DNA was electroporated into AM12 packaging cell line (Markowitz *et al*., 1988) using a gene pulser (BioRad, USA), and transfected cells were selected with 0.7 mg/ml of G418. G418 resistant colonies were pooled and used in subsequent experiments. Recombinant retrovirus was collected and used to infect CEM cells as described (Cho *et al*., 2001). Then, infected cell lines were cloned by G418 selection and limiting dilution.

Infection of CEM cells with ARV-2 virus

CEM cells (2×10^5) were infected with 100-1,000 50% tissue culture infective dose ($TCID_{50}$) units of ARV-2 isolated from chronically infected HUT78 cells in a volume of 1 ml in the presence of 4 μg/ml of polybrene (Sigma, USA). Cells were washed once and resuspended in the original volume. Every 3-4 days, a sample of cells was withdrawn for analysis and cells were passaged at a one to four dilution in fresh RPMI 1640 with 10% fetal bovine serum.

Extracellular p24 measurements antigen ELISA test

The concentration of HIV-1 p24 antigen was determined in 100 μl of cell-free supernatants using a p24 antigen specific ELISA kit (Dupont, USA) as stated in the manufacturer's instructions. Supernatants obtained at progressive times were serially diluted, and the p24 values were determined from the dilution yielding measurements on the linear part of a standard p24 curve.

Results and Discussion

Construction of RNA decoy encoding retroviral vectors

To determine the effectiveness of RNA decoy derivatives in inhibiting HIV-1 replication, we constructed tRNA expression cassettes that encode several different forms of RNA decoy sequences. The sequence and proposed secondary structure of the RNA decoys, tRNA expression cassette, and the retroviral vector utilized in this study is shown in Fig. 1. Fig. 1A(a) shows TAR RNA, which contains the entire TAR sequence required for inhibition of Tat (Sullenger *et al*., 1991). Tat binding region was indicated by the shaded circle. Fig. 1A(b) shows the minimal Rev binding domain (mRRE) RNA, which contains the 13 nt primary binding site for Rev found at the base of SLIIB domain of RRE forming the purine-rich “bubble” structure (shaded box) (Heaphy *et al*., 1991) and an artificial stem-loop (open box) that replaces the naturally occurring sequence in SLIIB of RRE. The artificial stem-loop sequence, 5′-CUUCGG-3′, is frequently found in ribosomal RNA and has been demonstrated to be highly stable (Tuerk *et al*., 1988; Cheong *et al*., 1990). The reason why mRRE was used rather than the full-length RRE sequence was that the short RRE-derived decoy may not bind to cellular factors and therefore, may be safer (Lee *et al*., 1994). To improve the RNA decoy activity, we constructed two modified RNA decoy derivatives that may contain the decoy function of both TAR and mRRE. One is a fusion RNA of TAR and mRRE (TAR+mRRE), where TAR RNA is located onto the mRRE RNA structure (Fig. 1A(c)). The other is an aligned RNA of TAR and mRRE (TAR-L-mRRE), where TAR RNA and mRRE RNA are split by an artificial linker sequence (L) (Fig. 1(d)).

The RNA decoys were expressed in the form of chimeric tRNA-RNA decoy transcripts (Fig. 1B). Various forms of RNA sequences shown in Fig. 1A were fused to the 3′ end of a wild type $tRNA_i^{met}$ gene (△3-2) that

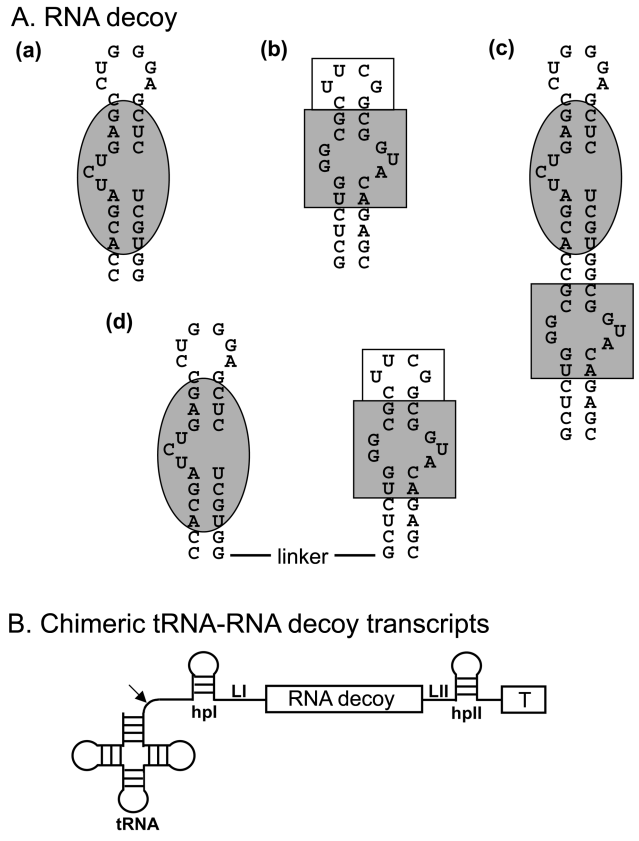

Fig. 1. Structure of chimeric tRNA-various forms of RNA decoy templates and retroviral vectors. A. The sequence of various forms of RNA decoys and their proposed secondary structures predicted by mfold computer simulating program (Jager *et al*., 1989). (a) TAR RNA (TAR). Primary Tat binding domain is in the shaded circle. (b) Minimal RRE RNA (mRRE). Primary Rev binding domain is shown in the shaded box, and a short stem-loop structure is in the open box, which replaces the naturally occurring stem-loop in RRE of the HIV-1. (c) Fusion RNA of TAR and mRRE RNA (TAR+mRRE). TAR RNA is fused onto the structure of primary Rev binding domain. (d) Aligned RNA of TAR and mRRE (TAR-L-mRRE). TAR RNA is linked upstream to mRRE RNA. Linker represents artificial sequence inserted between TAR and mRRE. B. The chimeric-various RNA decoy constructs consist of a wild type human $tRNA_i^{met}$ gene and a RNA decoy sequence inserted between the tRNA and the transcription termination signal (T). An arrow indicates the 3' end RNA processing site of the chimeric RNA. Two stem-loop structure (hpI and hpII) and additional short sequences (LI and LII) are present on both sides of the RNA decoy sequence in the chimeric tRNA-various transcript. C. The chimeric tRNA-variable RNA decoy constructs were inserted into the polylinker sequence present in the 3' LTR of the N2A vector to generate retroviral vectors. In the target cells, the tRNA-RNA decoy DNA template was duplicated and transferred to the 5' LTR.

retains a functional 3' RNA processing signal (indicated by an arrow). Hence, the chimeric transcripts could yield a liberated RNA decoy sequence. The RNA decoy sequences were constructed to harbor the additional two stem loop sequences (hpI and hpII) in the flanking region, which then would increase the stability of the liberated RNA decoy following process from the chimeric tRNA transcripts. In addition, these two stem loops were separated from the RNA oligonucleotide by short sequences, LI and LII, to minimize potential interference with Tat or Rev binding to the RNA decoy sequences. These modification of the tRNA structure resulted in a 5- to 20-fold intracellular increase in the expression and potency of RNA decoys relative to the unmodified tRNA cassette (Lee *et al*., 1994; 1995).

The tRNA-RNA decoy expression cassettes were inserted into the 3' LTR of the murine N2A retroviral vector. This was done as shown in Fig. 1C so that the tRNA-RNA decoy templates would be duplicated and present at both LTRs of the proviral DNA in the infected cells (Hantzopoulos *et al*., 1989). The names of the various retroviral vectors are DCT-mRRE, DCT-TAR, DCT-TAR+mRRE, and DCT-TAR-L-mRRE, respectively. Control vectors containing tRNA without an insert are termed DCT. Vector DNA was converted to the corresponding virus and used to infect CEM cells. G418-resistant clones were individually isolated by limiting dilution and expanded to clonal cell lines for further analysis. CEM cells that were transduced with DCT-RNA decoy derivatives were observed to express comparable decoy RNAs with northern analysis (data not shown).

Protection of CEM cell lines expressing RNA decoy derivatives from HIV-1

Parental non-transduced CEM cells and CEM cell clones, which were transduced with either control DCT vector or the various tRNA-RNA decoy-encoding retroviral vectors, were analyzed for their ability to support the replication of HIV-1. Cells were infected with 100-1,000 $TCID_{50}$ units of the ARV-2 virus, and the spread of virus in culture was first measured by determining syncytia formation (Table 1). We previously demonstrated that the syncytia formation pattern in CEM cells following HIV-1 infection directly correlated with the spread of virus in culture, which was measured by an extracellularly released p24 antigen concentration (Lee, 1998). Thus, syncytia formation will indicate HIV-1 replication in the virus-infected CEM cells. The expression of tRNA-RNA decoy transcripts in CEM cells had no measurable effect on their growth rate over an extended period time (data not shown). Also, microscopic examination revealed no morphological differences between parental CEM cells and the clonal isolates expressing the tRNA-RNA decoy transcripts even after extended culture period (Table 1).

The parental CEM cells and two clones transduced with the control DCT vector showed similarly strong syncytia formation following HIV-1 infection, which suggested

Table 1. Result of challenging tRNA-RNA decoy-expressing CEM cell clones with HIV-1

Cells	days PI					w/o I	Cells	days PI					w/o I
	d10	d13	d18	d24	d28	d28		d10	d13	d18	d24	d28	d28
CEM	+w	++	+++	++++	++++	-	DCT-TAR+mRRE						
DCT							-1	?	?	+w	++	++	-
-1	+	++	+++	++++	++++	-	-2	?	+	++	++	+++	-
-2	+w	+	++	+++	++++	-	-3	-	?	+	+	++	-
DCT-TAR							-4	-	-	+	+	++	-
-1	+	+	+	++	++	-	-5	+	+	++	++	+++	-
-2	+	+	+	++	++	-	DCT-TAR-L-mRRE						
-3	+	++	++	+++	++++	-	-1	-	-	-	-	+	-
-4	-	+	+	++	+++	-	-2	-	-	-	?	?	-
DCT-mRRE							-3	?	?	?	+w	+w	-
-1	-	-	+	++	+++	-	-4	-	-	-	-	?	-
-2	-	-	+	+	+	-	-5	-	-	-	-	-	-
-3	-	+w	+	++	++	-							
-4	-	-	-	-	+	-							

Patterns of HIV-1 replication in individual cells were determined by degree of syncytia formation in 10^5 cells at different days post-infection (PI) or 28 days without infection (w/o I). The numbers under the vectors indicate individual CEM cell clones. (-, no syncytia; ?, indeterminate syncytia; +w, less than 1% in 10^5 cells form syncytia; +, 1-10% form syncytia; ++, 10-15% form syncytia; +++, 25-50% form syncytia; ++++, more than 50% form syncytia.)

that the cells supported HIV-1 replication. CEM clones transduced with the DCT-TAR vector showed resistance against HIV-1 replication when compared with the control cells, but only transiently as shown by the increase in the syncytia formation at 24 days post infection. CEM cells transduced with DCT-mRRE vector showed more inhibition of syncytia formation than the control and clonal cells transduced with the DCT-TAR vectors. However, as previously reported (Lee, 1998), we observed some differences in the degree of HIV-1 replication among the CEM clones that expressed comparable levels of the same decoy RNA.

Contrary to our expectation, CEM cells transduced with DCT-TAR+mRRE, which might harbor both TAR and mRRE decoy function, showed only moderate inhibition of syncytia formation and showed even less than the cells transduced with DCT-mRRE. Several cellular factors have been demonstrated to bind TAR RNA sequence and interact with Rev and Tat proteins (Emerman and Malim, 1998). Thus, ineffective inhibition of HIV-1 in the cells expressing TAR+mRRE could be due to interference with simultaneous binding of Tat and Rev protein to a constrained and fused structure of TAR+mRRE RNA. Therefore, in order to exclude this potential limitation of RNA decoy structure for binding both the HIV-1 regulatory proteins and hence, maintain the decoy functions of both RNA decoys, we constructed a cassette that can express an aligned chimeric RNA, termed TAR-L-mRRE, where TAR RNA sequence was physically separated from mRRE sequence by artificial L sequence. In sharp contrast to any other cells, CEM cells transduced with DCT-TAR-L-mRRE showed very efficient inhibition of syncytia formation. Especially, all the cells transduced with this vector were the most durably protected against HIV-1, a result not seen in the other clones transduced with other vectors. These observations suggest that decoy function of both TAR and mRRE RNA could be sustained in the structural configuration of TAR-L-mRRE RNA. In conclusion, in the challenge experiments of clonal cell lines with HIV-1, coexpression of both TAR and mRRE RNA decoys in the form of TAR-L-mRRE RNA was found to be more effective in inhibiting HIV-1 than the expression of only one RNA decoy.

To confirm and evaluate the effectiveness of theTAR-L-mRRE decoy RNA in prohibiting HIV-1 replication, the spread of the virus in cells, transduced with DCT-TAR-L-mRRE, was compared to those with the control DCT vector and other decoy-encoding vectors through measurements taken by a p24 ELISA assay of the culture supernatant at 18 and 28 days post-infection (Fig. 2). As shown in the lanes 1 and 2, two CEM cells transduced with DCT supported the replication of HIV. CEM cells transduced with DCT-TAR showed only transient HIV-1 inhibition (lanes 3 and 4). Cells transduced with DCT-

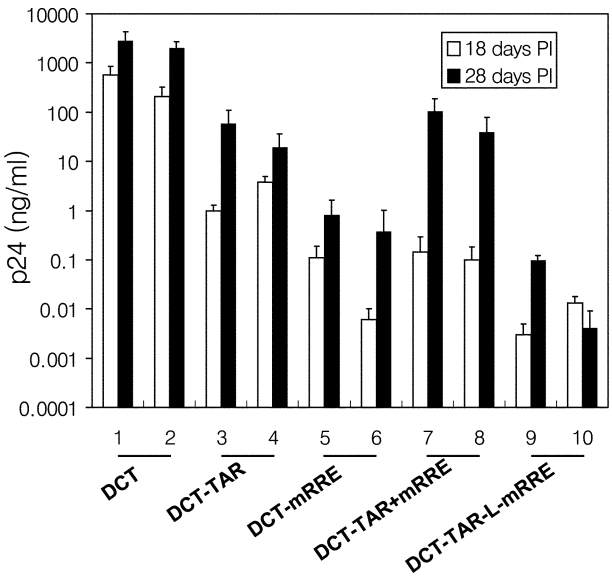

Fig. 2. HIV-1 secretion from CEM cells transduced with retroviral vectors encoding RNA decoy RNA. Two CEM cell clones transduced with DCT (clone No. 1 and 2 (lanes 1 and 2, respectively)), two CEM clones with DCT-TAR (clone No. 1 and 2 (lanes 3 and 4)), two CEM clones with DCT-mRRE (clone No. 2 and 4 (lanes 5 and 6)), two CEM clones with DCT-TAR+mRRE (clone No. 1 and 3 (lanes 7 and 8)), and two CEM clones with DCT-TAR-L-mRRE (clone No. 4 and 5 (lanes 9 and 10)) were infected with HIV-1, and secretion of the virus into the media 18 days and 28 days post infection was determined by a p24 ELISA test. The values shown represent the averages plus and minus standard deviation of three separate determinants.

mRRE showed more effective HIV-1 inhibition than those with DCT-TAR, but still not for a long period (lanes 5 and 6). In addition, TAR+mRRE decoy RNAs showed only moderate inhibition of HIV-1 replication to a degree less than mRRE decoy, which is in agreement with the result of syncytia formation (lanes 7 and 8). However, as shown in the lanes 9 and 10, p24 antigen secretion was very limited and reduced 10^4-10^5 fold in the case of all CEM cell clones expressing TAR-L-mRRE decoy RNA. Consequently, TAR-L-mRRE decoy RNA is a very potent antiviral agent against HIV-1.

Even though RNA decoys have been shown to efficiently inhibit HIV-1 replication in selected human CD4+ T cell lines, HIV-1 replication was inhibited at low levels and only transiently in bulk human peripheral blood CD4+ T cells that express the RNA decoys (unpublished data). Therefore, the effectiveness of RNA decoys in inhibiting HIV replication should be enhanced for their clinical application in HIV-1 gene therapy. In this study, we improved RNA decoy activity by co-expressing TAR and mRRE decoy RNAs in a single RNA molecule. This implies that there is synergy between the different gene therapy agents, as in the combined use of TAR and RRE decoys employed. Moreover, the possibility of HIV-1 escape from this combined decoy-mediated inhibition will be more unlikely, because it requires simultaneous mutation of TAR and RRE in a manner where both Tat and Rev preserve their respective functions, and this suggests that the combined decoy approach would be more effective. Another approach to develop potent anti-HIV-1 RNA decoys is to isolate RNA ligands, called RNA aptamers, that bind specifically and avidly to HIV-1 structural or regulatory proteins from RNA libraries through *in vitro* selection methods called SELEX (Tuerk and Gold, 1990; Bae *et al*., 2002). It will be interesting to determine the anti-viral validity of combined RNA decoy in future comparative studies with *in vitro* selected RNA aptamers.

To evaluate the efficacy ofTAR-L-mRRE decoy in clinical settings, polyclonal peripheral blood CD4+ T cells will be transduced with a vector expressing the decoy RNA and then challenged with HIV-1. In addition, the effectiveness of TAR-L-mRRE decoy RNA as a HIV-1 inhibitor will have to be compared with other potentially useful anti-HIV-1 agents that have recently been described, such as ribozyme, antisense oligonucleotides, or siRNA (Sullenger and Gilboa, 2002). To develop much more efficient anti-HIV-1 gene therapy protocols, the RNA decoy generated in this study could be combined with the other anti-HIV-1 agents as a single gene transfer vector. These studies together with the development of an efficient gene transfer system will have important implications for the design of clinically relevant gene therapy protocols against HIV-1, as well as other non-curable viruses.

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