Inhibition of human immunodeficiency virus type 1 replication by regulated expression of a polymeric Tat activation response RNA decoy as a strategy for gene therapy in AIDS

(simian immunodeficiency virus/tat/ribozyme)

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ABSTRACT We are investigating a strategy for somatic gene therapy to treat human immunodeficiency virus type 1 (HIV-1) infection by intracellular expression of an RNA decoy and a ribozyme. The RNA decoy, consisting of polymeric Tat activation response elements (TARs), is designed to compete for Tat binding in an equilibrium with viral TAR RNA, thereby inhibiting viral replication. The expression of polymeric TAR is regulated by the HIV long terminal repeat (LTR) and transcriptional activation is dependent on the presence of HIV Tat. Our initial studies indicated that plasmids expressing up to 50 tandem copies of TAR RNA (50TAR) inhibited tat-mediated gene expression by >90% in a transient transfection assay. A HIV LTR-driven 50TAR construct was subcloned into a replication-defective retroviral vector to ensure high-efficiency gene transfer into T lymphocytes. In addition, a gag RNA-specific ribozyme gene was introduced into the 50TAR containing retroviral vector to enhance the inhibitory effect of the construct (designated TAR-Rib). A human T-cell line (Molt3) was infected (transduced) with the TAR-Rib recombinant retrovirus and challenged with either HIV-1 or simian immunodeficiency virus (SIV). HIV-1 replication was inhibited by 99% in the TAR-Rib-transduced T cells and was maintained over a 14-month period, suggesting that this antiviral strategy suppresses the formation of escape mutants. Interestingly, the TAR-Rib also inhibited SIV replication in transduced T cells, which suggests that polymeric TAR is a general inhibitor of primate lentiviruses; therefore, the macaque model could be used for further in vivo testing of this antiviral gene therapy strategy.

The Tat protein is a potent transactivator of human immunodeficiency virus type 1 (HIV-1) gene expression (1) and may have additional functions in the pathogenesis of the acquired immunodeficiency syndrome (AIDS), including acting as a vascular cell growth factor, which may be relevant to HIV-associated Kaposi sarcoma (2–5). Therefore, Tat is a desirable target for an intervention therapy against HIV-1 infection. Tat activation of HIV-1 gene expression is dependent on a cis-acting element, the Tat activation response element (TAR), which extends from -17 to +80 in the HIV-1 long terminal repeat (LTR) (6). TAR RNA forms a stable stem-loop structure, and Tat binds to its “bulge” region with high efficiency (7). This RNA–protein interaction is essential for Tat activation of the HIV-1 LTR (8). Recently, we have demonstrated that a short polymeric TAR RNA (5 tandem copies) can inhibit Tat-mediated transactivation of the HIV-1 LTR in a transient transfection assay (9). Having validated this approach, we are attempting to develop this strategy further as a somatic gene therapy for the treatment of HIV-1 infection.

MATERIALS AND METHODS

Plasmid Construction. A blunt-ended fragment (Pvu II/Sca I), containing five tandem TARs, was isolated from plasmid LTR-5TAR (9) and ligated (100:1 ratio of vector to insert) into pSPT18 (Pharmacia). Clones containing multiple copies of the TAR insert (up to 45TAR) were isolated and the copy number and orientation were determined by restriction analysis. The polymeric TAR inserts (15TAR and 45TAR) were isolated from the pSPT18 constructs (Sal I/BamHI digestion) and subcloned into Sal I/BamHI-digested LTR-TAR-chloramphenicol acetyltransferase (CAT) vector, replacing the CAT sequence to generate LTR-20TAR and LTR-50TAR plasmids (see Fig. 1A).

Transient Transfection Assay. COS cells (3 × 10⁴ cells) were transfected with 2 μg of LTR-CAT and 0.3 μg of LTR-TAT, and cotransfected with 1 μg of pLTR-XTAR or control plasmids as described (9). CAT expression was determined as described (10).

In Situ Hybridization and Electron Microscopy. COS cells (5 × 10⁴ cells) were plated into chamber slides and transfected 24 hr later with a calcium phosphate precipitate of 1 μg of LTR-50TAR, 0.4 μg of pSVL-TAT (11), and 1.1 μg of pBR322. After 48 hr of incubation, cells were washed, fixed with methanol/acetone (1:1), and processed for in situ hybridization (12). A radiolabeled polymeric TAR DNA probe was used for hybridization under non-denaturing conditions, restricting the hybridization only to cellular RNA and not to DNA.

Four months after HIV-1 infection TAR-Rib (see below) and double-copy (DC) vector-transduced Molt3 cells were washed, fixed with 0.1 M sodium cacodylate and 1.25% glutaraldehyde, and examined by transmission electron microscopy.

Construction of the TAR-Rib Retrovirus Vector and Analysis by PCR. A PCR fragment containing a 65-bp gag-specific ribozyme was subcloned into the BamHI site of pRRE (13). Eight clones containing the ribozyme sequence were selected after restriction mapping. The orientation of the ribozyme in the pRRE-ribozyme construct was confirmed by an in vitro cleavage reaction (13) (see Fig. 2A).

The Xba I fragment (4.6 kb) containing LTR-50TAR was inserted into the SnaBI site of the DC vector to generate the

Abbreviations: HIV-1, human immunodeficiency virus type 1; LTR, long terminal repeat; TAR, Tat activation response element; SIV, simian immunodeficiency virus; RT, reverse transcriptase; FACS, fluorescence-activated cell sorting; CAT, chloramphenicol acetyltransferase; DC, double copy.

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DC-LTR-50TAR construct. The number of TARs was determined by restriction enzyme analysis. The RRE-ribozyme fragment (see Fig. 2A) was isolated from pRRE-ribozyme plasmid after HindIII and EcoRI digestion and cloned into the 3′-LTR site of the DC-LTR-50TAR plasmid. The resulting DC-LTR-50TAR-RRE-ribozyme was designated TAR-Rib (see Fig. 2B). The TAR-Rib plasmid was then transfected into the GPEC86 ecotropic packaging cell line (14). After 72 hr, the cell-free supernatant was used to infect the GP+envAm12 amphotropic packaging cell line (15). Three days later, cells were split and selected by using Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (FCS) and G418 sulfate (active weight, 0.5 mg/ml). G418-resistant colonies were pooled to produce replication-defective virus.

Genomic DNA was isolated from TAR-Rib-transduced or control Molt3 cells, and PCR was performed with neo gene-specific primers (16) to generate the 415-bp fragment. PCR was performed with the GeneAmp kit (Perkin-Elmer/Cetus) according to the manufacturer’s protocol.

HIV-1 and Simian Immunodeficiency Virus (SIV) Infection. TAR-Rib and DC vector-transduced Molt3 cells (2 × 10⁶ cells) were infected with 0.5 ml of fresh supernatant derived from Molt3 cells chronically infected with HIV-1ΔHIV, washed, and maintained in RPMI 1640 medium supplemented with 10% FCS and G418 sulfate (active weight, 1 mg/ml). The cultures were monitored for HIV-1 expression by using reverse transcriptase (RT) (17) and p24 ELISA (DuPont) according to the manufacturer’s protocol.

TAR-Rib and DC vector-transduced Molt3 cells (1 × 10⁶ cells) were treated with DEAE-dextran (20 μg/ml), washed, and infected with SIV-1mac251 at a low multiplicity of infection (ID₅₀, 0.5) for 2 hr at 37°C. The cells were washed and maintained in RPMI 1640 medium supplemented with 10% FCS. Supernatant samples were collected and assayed for the presence of SIV-1-p24 by ELISA (Coulter) according to the manufacturer’s protocol.

**RESULTS AND DISCUSSION**

Characterization of the Polymeric TAR Plasmids by a Transient Transfection Assay. Plasmids were constructed containing up to 50 copies of the TAR DNA under control of a HIV-1 LTR, which lacks the 5′ negative regulatory element (Fig. 1A). Deletion of the negative regulatory element does not influence the activation of the HIV-1 LTR by Tat, but it does allow higher levels of transcription compared to the wild-type LTR (18). Plasmids expressing polymeric TARs were cotransfected into COS cells with LTR-CAT and LTR-Tat. Fig. 1B demonstrates that plasmids containing either 20TAR or 50TAR inhibited tat transactivation of the HIV-1 LTR by >94% in this transient cotransfection assay. Under identical assay conditions, the maximal inhibition achieved using the original LTR-5TAR plasmid was only 75% (Fig. 1B). These results indicate that increasing the number of TARs results in a corresponding decrease in the level of Tat transactivation of HIV-1 LTR, suggesting an increased inhibitory effect on viral replication.

Intracellular localization of the polymeric TAR RNA in the transfected cells was determined by in situ hybridization. A radiolabeled polymeric TAR DNA probe was used under nondenaturing conditions for hybridization. These conditions restrict the hybridization of the probe to cellular RNA and minimize the binding to DNA. This analysis demonstrated that the polymeric TAR RNA was predominantly concentrated in the nucleus (Fig. 1C). The in situ localization of polymeric TAR RNA is consistent with our hypothesis that the decoy can compete with the HIV LTR for Tat binding in the nucleus (19), resulting in a substantial reduction in transactivation.

**Fig. 1.** (A) Schematic of polymeric TAR expression plasmids. (B) HIV-1 LTR-driven polymeric TAR plasmids inhibited tat transactivation of HIV-1 gene expression in a transient transfection assay in COS cells. LTR-CAT and LTR-TAT plasmids were cotransfected with equal concentrations of polymeric TAR and control plasmids (LTR-0TAR and 5TAR). Samples were assayed for CAT reporter gene expression as described (9). Plasmids expressing 20TAR or 50TAR inhibited tat transactivation of the HIV-1 LTR by 94%. (C) Intracellular localization of polymeric TAR RNA by in situ hybridization (12). The polymeric TAR RNA is localized predominantly in the nucleus of cells cotransfected with LTR-50TAR and pSVTAT. (×600.)

Generation of TAR-Rib Retroviral Vector and Transduced T Cells. To ensure high-efficiency gene transfer to the target cells, a replication-defective retroviral vector, based on Moloney murine leukemia virus, was used (kindly provided by E. Gilboa, Sloan-Kettering Institute for Cancer Research, New York). To achieve tat-inducible expression, the LTR-50TAR fragment was subcloned into the U5 region of the 3′ LTR of the DC retrovirus vector (20). Reverse transcription and provirus formation with this vector results in duplication of the inserted gene and, in this case, establishment of two copies of the LTR-50TAR gene for every integration event into the genome of the transfected cell. To further enhance the inhibitory effect of the retroviral construct, we introduced a gag RNA-specific ribozyme gene (13).
sequence. The fragment containing the gag-specific ribozyme (RRE-ribozyme) included a rev-responsive element to facilitate transport of the ribozyme RNA to the cytoplasm. Fig. 2A demonstrates the catalytic activity of the RRE-ribozyme gene in an in vitro RNA cleavage reaction. By introducing the RRE-ribozyme gene, we were attempting to generate a bifunctional construct that could (i) function in the nucleus as a Tat-binding TAR decoy, or (ii) function in the cytoplasm as a HIV RNA-specific ribozyme. We used this DC-LTR-50TAR-RRE-ribozyme construct, designated TAR-Rib (Fig. 2B), and the unmodified control DC vector to produce amphotrophic retrovirus. This recombinant virus was used to infect (transduce) a T-cell line (Molt3). Transduced cells were then selected with a neomycin analog (G418 sulfate) without single cell cloning. The presence of retroviral genomes was confirmed by PCR analysis (Fig. 2C).

Inhibition of HIV-1 Replication in the TAR-Rib-Transduced T Cells. After G418 selection, T-cell lines transduced with the TAR-Rib construct or the DC vector were challenged with HIV-1mb. During the first 20 days, HIV replication was significantly inhibited in the TAR-Rib-transduced T cells compared with the vector control (Fig. 3A). In both cell lines, however, viral replication ultimately resulted in observable cytopathic effects, characterized by a significant portion of cells forming syncytia. After 3 months of continuous culture, there was no further evidence of syncytia formation, although virus replication was still detectable by RT assay or p24 ELISA. We tested the p24 levels in the culture supernatants and cellular lysates of the TAR-Rib or DC vector-transduced Molt3 cells and compared them to the untransduced HIV-1-infected Molt3 control cells (Fig. 3B). Surprisingly, there was an unexpected reduction in the amount of p24 in the culture supernatants of the DC vector-transduced cells compared to the untransduced Molt3 cells. However, the amount of p24 detected in the cellular lysates was not significantly different. These results suggested that either the vector or the G418 selection has some effects on virion release. A comparison of the p24 (and RT) levels of the TAR-Rib-transduced cells with either the DC vector-transduced or untransduced Molt3 cells demonstrated at least 92% inhibition of HIV-1 replication. These data indicate that the TAR-Rib construct can function as a potent inhibitor of HIV-1 replication.

Four months after HIV-1 infection, a flow microfluorimetric analysis [fluorescence-activated cell sorting (FACS)] was performed on the TAR-Rib and vector-transduced T cells.

There was no detectable CD4 antigen on the surface of these cells. In contrast, >98% of the uninfected TAR-Rib or DC vector-transduced cells were CD4+, indicating that neither the retroviral vectors nor the G418 selection induced the loss of the expression of the CD4 antigen.

FIG. 2. (A) In vitro cleavage of HIV-1 RNA by the gag RNA-specific ribozyme. The RRE-ribozyme hybrid gene was cloned 3' of a T7 promoter. Eight plasmids containing an RRE-ribozyme sequence were tested for catalytic activity in vitro. The 291-nt substrate RNA was cleaved by the ribozyme, generating two products, P1 and P2. The HindIII/EcoRI fragment from the pRRE-ribozyme construct (plasmid 7) was subcloned into the retrovirus vector to generate the TAR-Rib construct. (B) Schematic representation of the integrated DC retrovirus vector (TAR-Rib). The advantage of the DC vector is that it integrates two copies of the TAR-Rib sequence into the genome of the transduced T cells. (C) Detection of retroviral vectors in transduced and G418-selected T cells. Molt3 cells were transduced with either TAR-Rib or DC vector and maintained in culture under G418 selection. PCR was performed on genomic DNA samples isolated from Molt3 cells (lane 2), DC vector-transduced cells (lane 3), and Tar-Rib-transduced cells (lane 4). Molecular size markers (lane 1) are φX174 Hae III fragments.
of CD4 receptors. After 14 months of continuous culture, inhibition of HIV-1 replication in the TAR-Rib-transduced T cells exceeded 99%. The residual low-level virus replication in the TAR-Rib-transduced T cells supported our hypothesis that the TAR RNA decoy competes for tat binding in an equilibrium with the proviral TAR RNA. In addition, we examined the cultures by transmission electron microscopy (Fig. 3C). Quantitative analysis of the number of viral particles showed abundant HIV-1 particles (typically 80–90 virus particles per cell) in the DC vector control cells and few mature particles (<10 virus particles per cell) in the TAR-Rib-transduced T cells.

Since we had already established that the gag RNA-specific RRE-ribozyme gene was active in vitro (Fig. 2A), a primer-extension experiment was designed to detect RNA cleavage products in the HIV-1-infected TAR-Rib-transduced T cells. However, the significant inhibition of HIV-1 replication by the TAR RNA decoy resulted in a highly reduced yield of viral RNA. Since the amount of the RNA cleavage product was difficult to detect at the given sensitivity of current assays, we do not know whether the addition of an anti-gag ribozyme gene augments the inhibitory activity of the LTR-50TAR construct.

Inhibition of SIV Replication in the TAR-Rib-Transduced T Cells. We were interested in determining whether the TAR-Rib retroviral construct could inhibit the replication of distantly related retroviruses. Therefore, we infected the TAR-Rib and vector-transduced Molt3 cells with SIVmac251. Up to 90% inhibition of SIV replication was observed in the TAR-Rib-transduced T cells when compared to the DC vector-transduced cells (Fig. 4), although the SIV infection did not result in syncytia formation. SIV-infected cells were also examined by electron microscopy. Quantitative analysis showed 50–75 viral particles per DC vector-transduced cell and 10–15 viral particles per TAR-Rib-transduced T cell.

CD4 expression was determined (FACS) on the SIV-infected TAR-Rib and DC vector-transduced cells. In contrast to the results of the HIV-1 infection, we found that 92% of the SIV-infected TAR-Rib-transduced T cells expressed CD4 on their surface. However, only 52% of the SIV-infected DC vector-transduced cells expressed CD4, suggesting that the TAR-Rib construct was able to protect cells from the loss of CD4 surface antigen induced by SIV infection. These results indicate that the TAR-Rib construct may inhibit SIV replication in T cells by sequestering the SIV Tat protein. This is consistent with reports that SIV tat can activate HIV-1 LTR-directed gene expression by interacting with the TAR element (21) and in agreement with the results of Sullenger et al. (22), demonstrating that a high-level expression of a single TAR RNA element from a tRNA promoter can inhibit both SIV and HIV-1 replication in vitro.

There are several possible explanations for residual HIV-1 replication in the TAR-Rib-transduced T-cell cultures. Low-level expression of the TAR-Rib construct may be insufficient to block HIV-1 replication during the initial phase of infection, resulting in a reduced but persistent virion release responsible for syncytia formation and cell death in HIV-1-infected Molt3 cells. This low-level expression may be due to either spontaneous mutations in the TAR-Rib construct during packaging as retroviral particles or possibly transcriptional interference from a cellular promoter occurring after integration (23). Another explanation could involve the Tat-dependent regulation of the TAR-Rib construct. Tat must be produced by the HIV-1 provirus to activate transcription of TAR-Rib from a HIV-1 LTR, and this will result in the release of some infectious HIV-1 particles. This persistent replication of HIV-1 in the TAR-Rib-transduced cells can induce syncytia formation with uninfected cells, resulting in a selective advantage for T cells expressing low levels of surface CD4 antigen. This may explain the results of the FACS analysis in which we observed a reduction in surface CD4 expression in TAR-Rib-transduced and HIV-1-infected Molt3 cells over time. Since there is no evidence for significant syncytia formation in vivo, we would not expect this selection to be a problem in our gene therapy strategy.

Somatic gene therapy is an exciting recent development in biomedical research (24, 25). We have shown that regulated expression of an RNA decoy can inhibit HIV-1 and SIV replication in T cells. The HIV-1 LTR-driven polymeric TARs can only be transcribed in the presence of Tat protein after virus infection. If the cell is not infected, the inhibitory gene will not be expressed. Regulation of gene expression in a gene therapy strategy is extremely important because we do not know that long-term constitutive expression of TAR or any other foreign gene will not be toxic to the cells. Combining the TAR decoys with anti-gag ribozyme gives an additional advantage to the inhibitory gene: one RNA transcript has dual function in blocking the activity of Tat protein and the translation of the gag mRNA. This inhibitory RNA blocks HIV-1 replication in T cells by >99% over a 14-month period in vitro in the absence of escape mutants, indicating that this strategy may have beneficial long-term effects in vivo. In addition, the demonstration of SIV inhibition opens the way for use of the SIV infection of macaques as a model for HIV-1 infection in humans.

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