Inhibition of Expression of HTLV-1 Structural Genes Mediated by Short Hairpin RNA *In Vitro*

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Abstract. *Background: Human T-lymphotropic virus I (HTLV-1) is associated with the T-cell malignancy known as adult T-cell leukemia/ lymphoma (ATLL) and with a disorder called HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Currently, the treatment of these diseases is based on symptom relief. RNA interference (RNAi) technology has been described as an efficient mechanism for development of new therapeutic methods. Thus, the aim of this study was to evaluate the inhibition of HTLV-1 structural proteins using short hairpin RNAs (shRNAs) expressed by non-viral vectors. Materials and Methods: Reporter plasmids that express enhanced green fluorescent protein-Gag (EGFP-Gag) and EGFP-Env fusion proteins and vectors that express shRNAs corresponding to the HTLV-1 gag and env genes were constructed. shRNA vectors and reporter plasmids were simultaneously transfected into HEK 293 cells. Results: Fluorescence microscopy, flow cytometry and real-time PCR showed that shRNAs were effective in inhibiting the fusion proteins. Conclusion: These shRNAs are effective against the expression of structural genes and may provide an approach to the development of new therapeutic agents.*

Isolated for the first time in 1979 from a T-cell leukemia-lymphoma patient (1), human T-lymphotropic virus type 1 (HTLV-1) indicated that human retroviruses could be related to cancer development, as later proposed by Gallo (2). Currently, it is known that HTLV-1 is the etiologic agent of adult T-cell leukemia/ lymphoma (ATLL) (3) and tropical spastic paraparesis or HTLV-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (4). Other inflammatory disorders including myositis, peripheral neuropathy, uveitis, arthritis, Sjögren syndrome and alveolitis have also been related to HTLV-1 infection (5). It is estimated that approximately 20 to 40 million people are infected worldwide. Although most infected individuals remain asymptomatic during their lifetime, 0.25% to 3.8% of them develop HAM/TSP and 2% to 3% develop ATLL. When all HTLV-1-related disorders are considered, approximately 10% of infected patients may develop these symptoms (5). Allogeneic stem cell transplantation, chemotherapy, nucleoside analogues, interferons and others strategies have been used to treat HTLV-1-related diseases, but with short-term benefits and, in some cases, a low survival rate (5-8). Thus, the treatment of HTLV-1-related diseases is basically limited to symptomatic relief.

In recent years, RNA interference (RNAi) has appeared as a potent and specific tool for gene silencing in mammalian cells, a property that may carry an enormous therapeutic potential (9). The RNAi mechanism is initiated when double-stranded RNAs (dsRNAs) expressed in or introduced into the cells are processed by Dicer, a ribonuclease III family enzyme, into 21-23 base pair RNAs (10). The processed dsRNAs (small interfering RNAs, siRNAs, or microRNAs, miRNAs) are associated with the RNA-induced silencing complex (RISC), an Argonaut family protein, in which the 21-23 bp molecules are processed into a single strand by dissociation or destruction of the sense strand. Thus, the RISC uses the antisense strand as a guide to recognize the messenger RNAs (mRNAs) in a sequence-specific manner and to repress their translation or induce their degradation (11). Intracellularly expressed short hairpin RNAs (shRNAs) can be used instead of siRNAs to obtain long-term gene silencing. These molecules (expressed by viral or non-viral vectors) are self-complementary RNAs that trigger the RNAi mechanism after their processing into siRNAs by Dicer (9).

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As is the case for other delta-type retroviruses, the HTLV-1 genome is flanked by the long terminal repeat (LTR) and contains *gag, pol* and *env* structural genes and, in contrast to the other retroviruses, also contains tax and rex regulatory genes (12). *gag* and *env* genes code for the precursor structural polyproteins of HTLV-1. The Gag precursor polyproteins are cleaved by viral proteases resulting in matrix (MA), capsid (CA) and nucleocapside (NC) proteins. In the same way, envelope precursor polyproteins are cleaved by cellular proteases, resulting in a mature Env protein composed of two subunits: a surface (SU) protein and a transmembrane (TM) protein (13, 14). While the Gag protein is related to virion assembly and release (13), the Env protein is involved in the interaction between the surface receptor GLUT-1 of the host cell and the virus (14).

Therefore, considering the great importance of structural proteins in viral infection, previous studies from our group using siRNAs (15) and the possibility to develop new therapeutic methods by silencing these genes, the aim of the present study was to evaluate the use of shRNAs expressed by non-viral vectors in transformed HEK-293 cells that express Gag and Env proteins.

Materials and Methods

Cell culture. The human embryonic kidney cell line HEK 293 was cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Gaithersburg, MD, USA) containing 10% heat-inactivated fetal bovine serum (Gibco-BRL) supplemented with L-glutamine (1 mM), streptomycin (100 μg/ml) and penicillin (100 U/ml) (Gibco-BRL) at 37˚C in a 5% CO_2 incubator.

Construction of reporter systems for gag and env. The construction and functionality of reporter systems was described previously (15). In brief, total RNA from HTLV-1-infected cells (MT-2, ECACC, Salisbury, Wiltshire, UK) was extracted and reverse transcribed using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). The *gag* and *env* cDNAs were amplified by PCR and resulting DNA fragments were digested with *Hind* III and *Bgl* II to be inserted into the multi-cloning site of the pEGFP-C1 expression vector (Clontech, Mountain View, CA, USA). These resulting plasmids (with the *EGFP* gene located upstream of the target gene) were named pEGFP-Gag and pEGFP-Env. The functionality of the reporter systems was analyzed by immunocytochemistry assay and confocal microscopy.

Construction of shRNA vectors. The shRNA vectors (pSilGag1 and 2, pSilEnv1 and 2, and pSilScramble) were constructed using the pSilencer 5.1 H1 Retro kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. The target regions of the genes were chosen using the siRNA Target Finder program (Applied Biosystems), and our previous results (15). The sequence targets are presented in Table I.

Transfection of HEK 293 cells. Forty-eight hours before transfection, 2×10^5 cells were seeded to 80% confluency in 6-well culture plates with DMEM (2 ml/well). A total of 5 μg of reporter plasmid and 5 μg of shRNA vector were mixed with SuperFect Transfection Reagent (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. Forty-eight hours after transfection, the expression of the EGFP-Target (Gag or Env) fusion protein was observed directly under an inverted fluorescence microscope (Olympus IX71, Tokyo, Japan) and RNA was extracted. This protocol was performed in duplicate.

Table I. *Vector names and corresponding target sequences for gag and env genes.*

Vector name	Gene target	Gene position	Target sequence
pSilGag1	*gag*	609-629	5'-gcagctagatagccttata-3'
pSilGag2	*gag*	968-988	5'-gccctctaggagatatgtt-3'
pSilEnv1	*env*	659-679	5'-gcactaattatacttgcat-3'
pSilEnv2	*env*	1168-1188	5'-ggaggattatgcaaagcat-3'
pSilScramble	-	-	Ambion Cat#AM5782

Fluorescence microscopy. The fluorescence of EGFP target in transfected HEK 293 cells was observed 48 hours after transfection under an inverted fluorescence microscope by exciting the cells at 488 nm. The light microscopic view of cells and fluorescence images in the same field were captured.

Flow cytometry analysis. Forty-eight hours after transfection, the cells were trypsinized, harvested and washed with phosphate-buffered saline, pH.7.5 (PBS). The cell pellet collected after centrifugation at 1200 rpm for 5 minutes at 4˚C was resuspended in PBS to measure fluorescence using a BD FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA) with filters (emission, 507 nm; excitation, 488 nm). Non-transfected HEK 293 cells were used as control. The data collected by flow cytometry were analyzed with the CellQuest software (BD Biosciences). The mean fluorescence intensity of the cell population that exceeded the fluorescence intensity of control cells was calculated.

Real-time quantitative PCR. One microgram of RNA was reverse transcribed into cDNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's recommendations. PCR amplification was carried out in 96-well plates with optical adhesives using Taqman Universal PCR master mix (Applied Biosystems), specific probes (FAM and MGB labeled) and specific primers for *gag* and *env* genes. For real-time quantitative PCR, the sense and antisense primers for the *env* gene were 5'-TCTAGTCGACGCTCCAGGATATG-3' and 5'-CAGTTGGCTGG GTTCGGTAT-3', respectively. The sense and antisense primers for the *gag* gene were 5'-CCCCCAAGTTCTTCCAGTCA-3' and 5'-TGCCATGGGCGATGGT-3'. The probes carried FAM as the 5' reporter dye and MGB as the 3' quencher dye. The probe sequences were 5'-CCCCATCTGGTTCCT-3' and 5'-CCACATGGTGCCCC-3' for the *env* and *gag* genes, respectively. The Human GAPD (GAPDH) Endogenous Control (Applied Biosystems item number 4310884E) was used, and the relative expression was calculated by the ddCT method (16, 17).

Statistical analysis. Flow cytometric data are reported as the mean±SD. Statistical analysis was performed by one-way analysis of variance (ANOVA). Student's *t*-test was also applied to determine

which means were significantly different ($p<0.05$) from the control mean. All analyses were carried out using the GraphPAd Prism software package (GraphPad software, San Diego, CA, USA).

Results

Fluorescence microscopy. Forty-eight hours after transfection, fluorescence microscopy showed that pEGFP-Gag/pSilGag1 co-transfected cells presented reduction of EGFP expression compared to pEGFP-Gag/pSilScramble co-transfected cells and pEGFP-Gag transfected cells. The same profile was observed for pEGFP-Gag/pSilGag2 co-transfected cells, but at a lower intensity (Figure 1A). The pEGFP-Env/pSilEnv1 co-transfected cells also presented reduced fluorescence compared to pEGFP-Env/pSilScramble co-transfected cells and pEGFP-Env transfected cells (Figure 1B).

Flow cytometry. Flow cytometric analysis was performed and showed that pEGFP-Gag/pSilGag1 co-transfected cells presented 65% and 59% lower mean fluorescence than pEGFP-Gag/pSilScramble and pEGFP-Gag transfected cells, respectively ($p<0.05$). The pEGFP-Gag/pSilGag2 co-transfected cells presented lower fluorescence (19%) than pEGFP-Gag/pSilScramble ($p<0.05$) and a small (6%) difference compared to pEGFP-Gag transfected cells (Figure 1C). Mean fluorescence reductions of 74% and 70% were also observed in pEGFP-Env/pSilEnv1 and pEGFP-Env/SilEnv2 co-transfected cells compared to pEGFP-Env/pSilScramble co-transfected cells, respectively ($p<0.05$). When pEGFP-Env/pSilEnv1 and pEGFPEnv/pSilEnv2 co-transfected cells were compared to pEGFP-Env transfected cells, fluorescence reductions of 44% and 35% were observed, respectively ($p<0.05$) (Figure 1D). Intriguingly, for both experiments the pEGFP-Gag and Env/pSilScramble co-transfected cells exhibited greater EGFP expression than pEGFP-Gag or pEGFP-Env transfected cells.

Real-time PCR. Real-time PCR evaluated the silencing of *gag* and *env* mRNA and showed a reduction of 21% and 27% in mRNA levels for pEGFP-Gag/pSilGag1 and pEGFP-Gag/pSilGag2, respectively, compared to pEGFP-Gag/pSilScramble co-transfected cells. Decreased expression was observed for both co-transfected cells compared to pEGFP-Gag transfected cells, with a reduction of 15% for pEGFP-Gag/pSilGag1 and a reduction of 21% for pEGFP-Gag/pSilGag2. No difference was observed when pEGFP-Gag and pEGFP-Gag/pSilScramble transfected cells were compared (Figure 1E). For *env* mRNA, a reduction of 12% was observed for pEGFP-Env/pSilEnv1 and pEGFP-Env/pSilEnv2 co-transfected cells compared to pEGFP-Env/pSilScramble co-transfected cells, while a reduction of 8% was observed for pEGFP-Env/pSilEnv1 and pEGFP-Env/pSilEnv2 co-transfected cells compared to for pEGFP-Env transfected cells (Figure 1F).

Discussion

Previous studies have shown that shRNAs are more efficient than siRNAs, possibly explained by the fact that shRNAs are processed by Dicer into siRNAs to become effective, and this processing favors the incorporation into the RISC complex (18, 19). Thus, we tested the possibility of using vectors that express shRNA to inhibit the HTLV-1 structural genes in HEK 293 co-transfected cells.

In fact, compared to results obtained previously with siRNAs (15), the flow cytometry data showed that the reduction of Env protein was stronger with the use of vectors that express the corresponding shRNA. Intriguingly, this effect was not observed for Gag protein. Fluorescence microscopy and, to a lesser extent, real-time PCR also showed that vectors expressing shRNAs were effective at gag and env gene silencing. The lower reduction observed by real-time PCR compared to flow cytometry can be explained by the possibility that processed shRNAs do not bind to target mRNA with total complementarity. Thus, even though mRNA is being silenced by translation repression, it does not suffer degradation and can be detected by real-time PCR.

Previously, our group showed the efficient inhibition of *gag* and *env* genes by siRNAs but the inhibition levels varied depending on the siRNA designed (15). It is possible that similar differences among the effects of vectors expressing shRNAs could be observed. This may be explained by the fact that one strand of siRNA remains preferentially bound to the RISC complex. Instead of the sense strand being degraded, the antisense strand is degraded and, consequently, mRNA inhibition is not observed. On the other hand, the complementarity-determining region may be inaccessible to the antisense strand because of the intrinsic characteristics of mRNA (20).

Viral infections are treated with many different strategies, but the treatments employed for some viruses have not yielded good results. In HTLV-1 infection specifically, the treatment is based on symptom relief. Due to the lack of alternatives for the effective treatment of HTLV-1 infection, the use of RNAi technology may be important for the development of new therapeutic protocols. The RNAi-based strategy has been employed *in vitro* to inhibit viral replication since the first study with respiratory sincitial virus was published (21). These studies showed that RNAi can be used to protect the cells against viral infection by inhibiting viral antigens and accessory genes, controlling the transcription and replication of the viral genome, and preventing virus assembly and virus-host cell interaction (22). RNAi has been recently used against viral infection *in vivo*. Phase I and II clinical trials are currently underway and have shown that RNAi is well tolerated (9).

The inhibition of HIV-1 replication by RNAi techniques has been widely studied *in vitro*. These studies include the

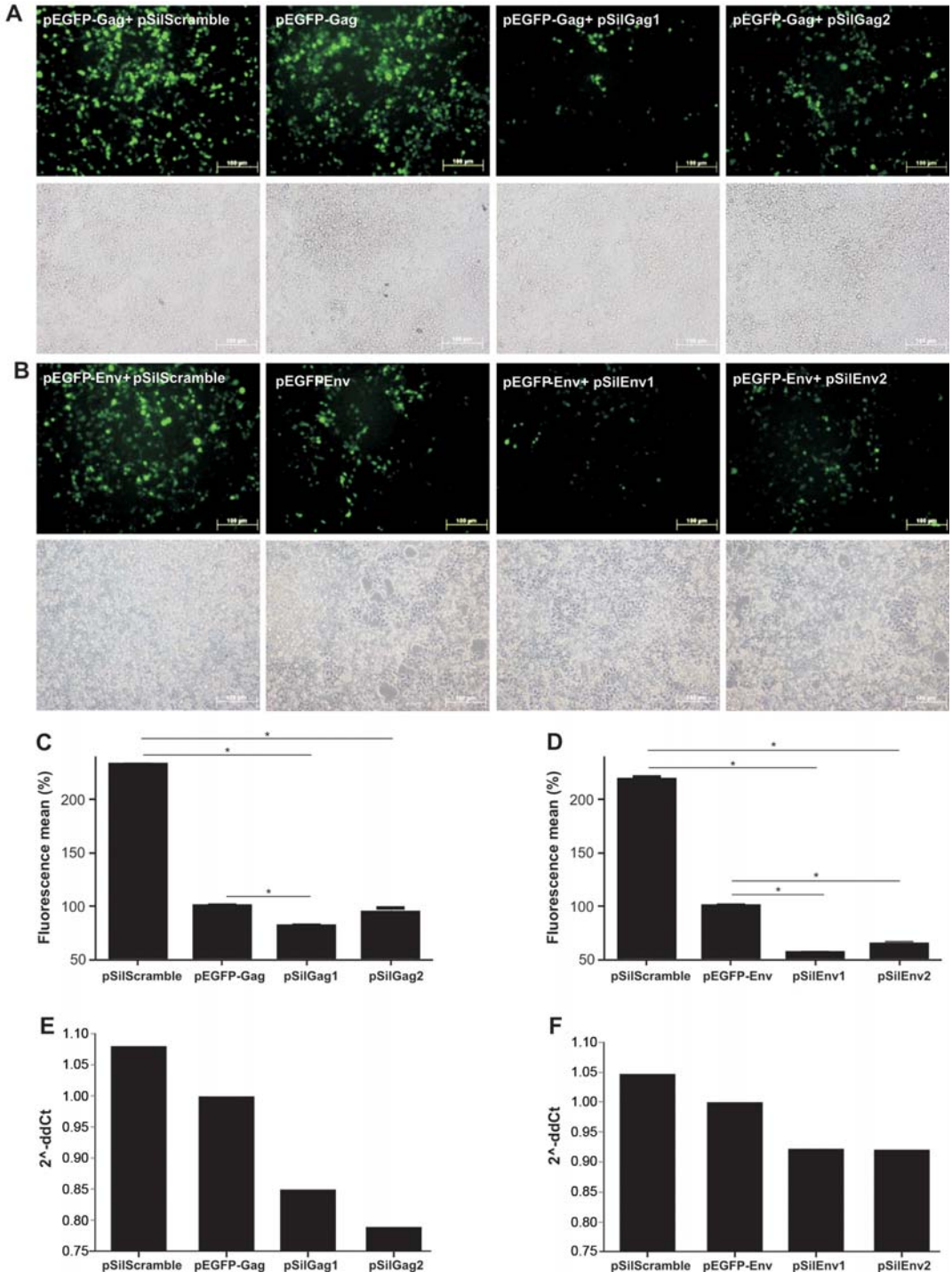

Figure 1. *EGFP-Gag and EGFP-Env expression in transfected HEK 293 cells. A: Fluorescence microscopy of cells co-transfected with pEGFP-Gag/pSilScramble, pEGFP-Gag, pEGFP-Gag/pSilGag1 and pEGFP-Gag/pSilGag2. B: Fluorescence microscopy of cells co-transfected with pEGFP-Env/pSilScramble, pEGFP-Env, pEGFP-Env/pSilEnv1 and pEGFP-Env/pSilEnv2. The upper panels represent the cell fluorescence images recorded 48 hours post-transfection and the lower ones represent the light microscopic view of the same field (×100). C: Mean fluorescence intensity of cells transfected with pEGFP-Gag alone and co-transfected with pEGFP-Gag/vectors measured by flow cytometry; the mean and standard deviation of two independent experiments are shown. D: Mean fluorescence intensity of cells transfected with pEGFP-Env alone and co-transfected with pEGFP-Env/vectors measured by flow cytometry; the mean and standard deviation of two independent experiments are shown. E: Relative mean expression of the gag gene in cells transfected with pEGFP-Gag and co-transfected with pEGFP-Gag/vectors. F: Relative mean expression of the env gene in cells transfected with pEGFP-Env and co-transfected with pEGFP-Env/vectors.*

efficient silencing of structural genes (*gag, env* and *pol*), regulatory genes (*tat*, *vif* and *rev*) and host genes (23). Similarly to HIV-1, RNAi was also employed in HTLV-1 studies to demonstrate the main HTLV-1 receptor (14), some regulatory *tax* gene functions (24,25), virus release (26), and host cell gene function (9), but there is no report on the use of shRNAs for HTLV-1 structural genes.

The present study is the first to demonstrate the use of shRNA vectors to inhibit the structural genes of HTLV-1. Our results show that specific shRNAs decrease the levels of HTLV-1 mRNAs and structural proteins, indicating that RNAi technology could be used as a tool to study the pathways that involve these proteins and also as a potential tool for the development of new RNAi-based therapeutic drugs against HTLV-1 infection.

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