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Triple combination lentiviral vector-based haematopoietic stem cell gene therapy for inhibition of drug-resistant HIV-1

Key Messages

1. A triple combination lentiviral vector Lenti-TriC was successfully constructed, in which lhRNA targeting HIV-1 was driven by the U6 promoter, shRNA targeting CCR5 was driven by the H1 promoter, and TRIM5α and EGFP were bicistronically expressed under the control of EF-1α promoter.
2. Lenti-TriC expressed lhRNA (targeting HIV-1) and inhibited HIV-1 gene expression. Lenti-TriC expressed shRNA (targeting CCR5) and significantly inhibited CCR5 gene expression.
3. Lenti-TriC inhibited HIV-1 replication in human CEM T cells, primary PBMC cells, and macrophages.
4. Lenti-TriC did not affect the differentiation potential of CD34+ cells.
5. Lenti-TriC could be used for haematopoietic stem cell gene therapy to inhibit HIV-1 replication.

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Introduction

Although current combination therapies significantly decrease mortality, novel approaches are needed to prevent the emergence of resistant HIV-1 variants. RNA interference (RNAi) is a promising modality for inhibition of HIV-1 RNAs. The use of a single RNA (siRNA) or short hairpin RNA (shRNA) targeting viral and host proteins is reported to inhibit HIV-1 replication.[1] However, the use of siRNA or shRNA is limited owing to the emergence of RNAi-resistant escape viruses.[2] To avoid escape from RNAi, the virus should be simultaneously targeted by multiple shRNAs. Long hairpin RNAs (lhRNA) can be used to produce multiple effective siRNAs.[3,4]

Mammalian cells have developed diverse strategies to restrict retroviral infection. Tripartite motif-containing 5 isoform-α protein from rhesus monkey (TRIM5αrh) restricts HIV-1 infection at a postentry, preintegration stage in the viral life cycle.[5] Stem cell–based gene therapy of HIV infection aims at inhibiting HIV replication and the progression to AIDS by the introduction of antiviral genes (eg siRNA) in primitive haematopoietic stem cells. To obtain sustained transgene expression and anti-viral protection in differentiated HIV-susceptible end-stage cells such as T cells and macrophages, CD34+ cells are widely used for gene transduction.[1,5] Lentivirus is an attractive vector for transducing CD34+ cells based on its high transduction efficiency and long-term expression of transgenes. We aimed to develop a triple combination lentiviral vector-based haematopoietic stem cell gene therapy for inhibition of HIV-1.

Methods

This study was conducted from January 2009 to July 2011.

Construction of a triple combination lentiviral vector

Lenti-TriC was constructed by multiple step cloning. For the construction of pU6-lhHIV, the U6 promoter, with a CalI site at its 5'- end, was cloned into a pBluescriptKS(+) vector, bridging with lhHIV by a SalI site. The lhHIV sequence ended with an EcoRI site. For the construction of pH1-shCCR5, the H1 promoter, with a ClaI site at its 5'- end, was cloned into a pBluescriptKS(+) vector, bridging with shCCR5 by a Bgl II site. The shCCR5 sequence ended with an EcoRI site. The EF-1α promoter was linked at the 5'- end of chimeric TRIM5α genes by Kozak sequence (5'- CGCTAGCGCTACCGGTCGCCACC-3'). The EF1α-TRIM5α was then cloned into pIRES2-EGFP by the 5'-EcoRI site and the 3'-SalI site. The EF1-TRIM5-IRES2-EGFP fragment was then obtained by restriction enzyme digestion at the XbaI and EcoRI sites.

The pU6-lhHIV was digested at ClaI and SmaI sites to obtain the U6-lhHIV fragment. The pH1-shCCR5 was digested by ClaI, and then blunted by polymerase, followed by digestion on the EcoRI site. The H1-lhCCR5 was released with the 5'- blunt end and the 3'-EcoRI site. The SmaI site of U6-lhHIV was ligated with the 5'- blunt end of H1-shCCR5 fragment, resulting in a U6-shHIV-H1-shCCR5 fragment with a 5'-ClaI site and a 3'-EcoRI site. The U6-

lhHIV-H1-shCCR5 fragment was cloned into the pSL6 vector at the ClaI and EcoRI sites.[6] The EF1alpha-TRIM5-alpha-IRES2-EGFP fragment was cloned into the pSL6-U6-lhHIV-H1-shCCR5 fragment on its EcoRI and NheI sites, yielding the final product Lenti-TriC.

Lentivirus production and transduction

The VSV-G-pseudotyped lentiviruses were produced by cotransfecting HEK293T cells with the transfer vector and three packaging vectors: pMDLg/pRRE, pRSV-REV, and pCMV-VSVG. Subsequent purification was performed using ultracentrifugation. For target cell transduction, 1000 cells were plated in 24-well plates and were transduced with lentivirus in the presence of 8 μg/mL polybrene (Sigma). The culture medium was replaced after incubation for 12 hours. Transduced cells were sorted by means of fluorescence-activated cell sorting before use, when the transduction efficiency was less than 70% (based on enhanced GFP expression).

Isolation and culture of primary human cells

Human peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation in Ficoll-Hypaque (Sigma, St. Louis, MO, USA) from buffy coats of HIV-1-seronegative individuals. They were maintained in RPMI-1640 medium supplemented with 10% FBS in the presence of 2 mM L-glutamine, 1% Penstrep, and then stimulated with 5 μg/mL phytohaemagglutinin (Sigma) and 200 U/mL interleukin-II (Invitrogen, USA) for 3 days before transduction with lentiviral vectors. Monocyte-derived macrophages were obtained from PBMCs by adherence to plastic for 12 hours in DMEM supplemented with 10% human serum (Cellgro, Herndon, VA, USA). They were then washed and cultured in the presence of macrophage colony-stimulating factor in 2 ng/mL DMEM (M-CSF, Sigma) for another 7 to 10 days, enabling the cells to fully differentiate before infection. The medium was replaced twice during the incubation period. The primary cells were maintained at 37ºC with 5% CO_2 in a humidified incubator.

Generation of infectious HIV-1

Infectious HIV-1 particles were generated using the proviral DNA construct pNL4-3 obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. Two million HEK293T cells were transfected with 5 mg of proviral DNA using lipofectamine 2000 (Invitrogen). The virus titre was analysed for HIV-1 p24 antigen using an enzyme-linked immunosorbent assay (Beckman Coulter, Fullerton, CA, USA). The p24 values were calculated using a Dynatech MR5000 enzyme-linked immunosorbent assay plate reader (Dynatech Lab, Chantilly, VA, USA).

HIV-1 challenge and antiviral assays

Cells were infected with vector or Lenti-TriC and then expanded for 72 hours. They were then challenged with recombinant HIV-1 NL4-3 virus at a multiplicity of infection of 0.01. After overnight incubation, the cells were washed three times with Hank's balanced salts solution and cultured using the Roswell Park Memorial Institute's 1640 medium with 10% foetal bovine serum. At designated time points, culture supernatants were collected and analysed for HIV-1 p24 antigen using ELISA (Beckman Coulter, Fullerton, CA, USA). The p24 values were calculated.

Results

Construction of a triple combination lentiviral vector

We constructed a triple combination lentiviral vector (Lenti-TriC) targeting three stages of HIV-1 life cycle. Lenti-Tri-C contained a lhRNA targeting a conserved, untranslated region of HIV-1, the shRNA targeting CCR5, TRIM5α, and EGFP cDNAs bicistronically under the control of the EF-1α promoter (Fig 1).

We designed a 65 bp length hairpin RNAs targeting conserved HIV-1 untranslated 5' long terminal repeat for generating two effective shRNAs to inhibit HIV-1. This overcame the emergence of RNAi-resistant HIV-1, which is a frequent consequence after a single shRNA treatment. Lentiviral vector integrated into the host genome after transduction and enabled long-term expression of shRNAs and antiviral genes. Lentiviral vector could efficiently transduce CD34+ cells and offer the feasibility to transduce a large quantity of CD34+ cells for clinical application. Moreover, the anti-HIV effect mediated by CCR5 inhibition and overexpression of TRIM5αrh was independent of the HIV RNA sequence, which was essential to obtain sustained anti-HIV efficacy, owing to the high mutation rate of the HIV-1 genome. Most importantly, the combination of lhRNA against HIV-1, shRNA targeting CCR5 and TRIM5αrh cDNA into one single lentiviral construct inhibited HIV-1 at three different stages of its lifecycle: entry, postentry and preintegration, and replication.

Inhibition of HIV-1 replication in primary human PBMCs and macrophages

To determine whether human PBMCs could be protected by the transduction of Lenti-TriC vectors, we isolated PBMCs from HIV-1-seronegative individuals, doubly transduced the PBMCs with lentiviral vectors, and then challenged the cells with HIV-1 NL4-3 virus. We observed a potent protective effect estimated to be a 9-fold reduction of p24 antigen levels in T cells transduced with Lenti-TriC at day 7 after challenging with HIV-1 NL4-3 virus (Fig 2). Notably, a positive protective effect was detected during almost the entire month of monitoring.

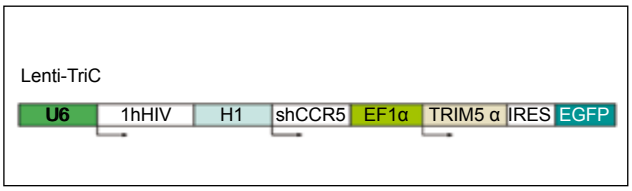

Fig 1. Schematic diagram of Lenti-TriC

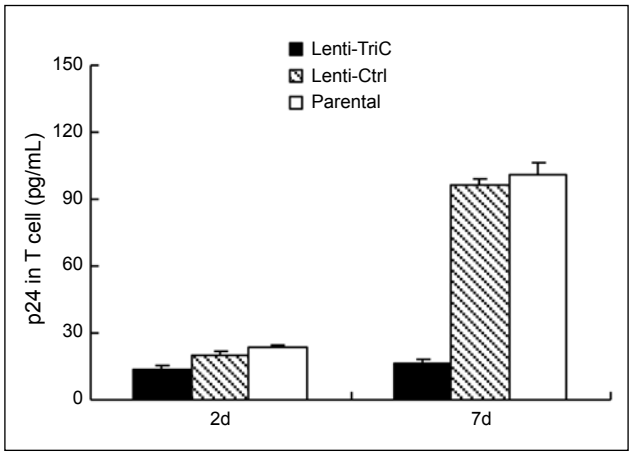

Fig 2. Lenti-TriC inhibited HIV-1 replication in human primary peripheral blood mononuclear cells

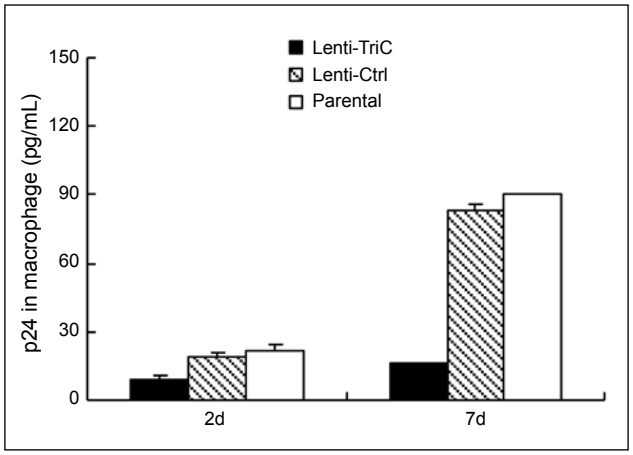

Fig 3. Lenti-TriC inhibited HIV-1 replication in human primary macrophages

To test the inhibitory effect on HIV-1 replication in human non-proliferating macrophages, these primary cells were transduced with the lentiviral vectors. These macrophages transduced with lentiviral vectors were then challenged with HIV-1 NL4-3 virus. During the 7 days of monitoring of these human macrophage challenge experiments, inhibition of HIV-1 replication was detected in the Lenti-TriC group (Fig 3). Notably, transduction efficiency with these SNV vectors for these primary human cells was approximately 30 to 40%.

Discussion

Many gene therapeutic constructs with different mechanisms of action have been tested individually in both T-cell lines and haematopoietic stem cells. Owing to the complexity of the lifecycle and the high mutation rate of the HIV genome, the use of a single anti-HIV gene therapeutic construct is not adequate to afford extended viral protection.

We developed a triple combination lentiviral vector Lenti-TriC that targeted three stages of the lifecycle for the HIV gene in the haematopoietic stem cell setting. This lentiviral vector generated (1) lhRNAs targeting a conserved untranslated region of HIV-1, (2) shRNAs targeting CCR5, and (3) expressed TRIM5α. We tested the anti-HIV efficacy of Lenti-Tri-C in CEM T cells, human primary PBMCs, and macrophages. We also examined the usefulness of Lenti-Tri-C for HIV gene therapy by transducing CD34+ cells. Our results pave the way for the development of lentiviral vector-based hematopoietic stem cell gene therapy to inhibit HIV-1. However, all the data were based on in vitro cell culture studies; this was the limitation of this project. The main difficulty in carrying the experiments (and future animal/clinical studies) is to prepare sufficient amount of pure lentivirus.

Acknowledgement

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