Original Article

Cloning, expression and library construction for HIV-1 Tat Protein

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Abstract

Background: Designing novel therapeutic agents has been a critical challenge for HIV disease.
Materials and Methods: In current study a DNA sequence which was encoded the Tat protein was synthesized and inserted in pET 28 vector. Vector was cloned in BL21-DE3 E. coli and cultured in TB media. After protein expression, recombinant Tat protein was purified by NTA affinity chromatography. The Tat purified protein efficiency and confirmed by SDS-PAGE and Western blot, respectively. We were immunized the camel against HIV-1 Tat recombinant protein to made a camelid antibody library. Total RNA was extracted from camel lymphocytes and VHH fragments synthesized and amplified using RT-PCR and Nested- PCR methods by special primers.
Results: The 350- 450 bp VHH gene fragment was produced by RT-PCR and Nested- PCR and extracted from agarose gel 1%. Then gel extraction was performed and pure fragments were inserted in HEN-4 vector by T4 DNA ligase.
Conclusion: The library can be applied for biopanning and isolation of nanobody against HIV-1 Tat Protein. Nanobody small size may be a useful drug for treatment of HIV disease because give them the potency of the recognizing the cryptic epitopes of tat and neutralized the virus.
Keywords: HIV-1, Tat Protein, VHH, Nanobody

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Introduction

The Human immunodeficiency virus (HIV) is caused the acquired immunodeficiency syndrome (AIDS). HIV type 1 (HIV-1) is one of types of this retroviral complex. There are many transactivatiors which regulate transcription of HIV-1 in cellular or viral manner. Tat is a protein which regulates and enhances the efficiency of transcription of the HIV dsDNA. Tat gene encodes Tat protein in HIV-1 (1). Tat represents for "Trans-Activator of Transcription" an constitutes of 86or 101 amino acids depending on the virus subtype (2, 3). TAR (Transactivation response element) an untranslatable sequence of all HIV RNAs which is located on 5' region is necessary for mediation of Tat transactivation (4, 5). TAR element prepares binding site for Tat in the nearside ofHIV-1 promoter (6).

Tat mediated transcription needs interaction between Tat and Tat binding factors –small proteins- which causes protein phosphorylation when some cellular proteins are attached to TAR region(7, 8). Tat acts as a transcription initialization factor (TIF) and increases the transcription elongation (9). Also, Tat plays a critical role in the HIV disease process. Tat protein is released by infected cells in culture media, and can be measured in the blood of HIV-1 positive patients which is a profitable resort for surpassing the body's response (10). It has been showed that Tat protein is able to stimulate the expression of tumor necrosis factor

α and ß , IL-10and IL-2 (11-13) which they may bean activator of expression and replication of HIV-1 gene (14, 15). Tat only inhibits PHA (phytohemagglutinin)-a mitogenic lectin-induced proliferation of primary human lymphocytes but also decrease the expression of MHC class I genes (16). Furthermore Tat can enhances the expression of cytokine transforming growth factor ß1 (TGF-ß1) which the potentially is an immunosuppresor agent (17). It is believed that HIV-1-infected cells have the ability of recognition of specific cytotoxic T lymphocytes and avoiding the immune surveillance (18, 19).

Scientists have investigated the inhibition of Tat and suggested that some Tat antagonists might be useful in treatment of HIV infections (20, 21).The newly introduced treatment for HIV-1 infection is employing of nobodies. Nanobody is a single-domain antibody which is consisting of hyper variable domain (VHH) of heavy chain camel immunoglobulin. Like a whole antibody, it is able to bind selectively to a specific antigen. They weight 11–15 kDa and can be produced naturally in Camelid such as camels and llamas. There have desired features like: physical stability, high tissue penetration, increased solubility, and low immunogenicity. Also nanobodies could be easily generated in multivalent forms by using short suitable peptide linkers (22). In this study we want to clone and express HIV-1 Tat Protein and injecting the Tat protein in camel to isolate the specific nanobody.

Methods

Ethics Statement. All used procedures in this study were approved by the Institutional Ethical Committee and Research Advisory Committee of Pasteur Institute of Iran based on National Specific Ethical Guidelines for Biomedical Research issued by Ministry of Health and Medicinal Education (MOHME) of Iran in 2005.

Cloning and expression of Tat gene. First Tat-HIV gene was cloned on pET 28 vector and transformed to E. coli BL21-DE3 bacteria. For confirmation of cloning Tat gene containing vector was digested with *NcoI* and *XhoI* restriction enzymes.

Analysis of protein Expression via SDS-PAGE and western blot. The transfected bacterium was incubated at 37°C with shaking speed 180rpm. Tat protein expression was investigated in different time points: 30 min. 1, 2, 3, 4, 24 hours. Different samples were gathered before and after protein induction to perform SDS-PAGE. The SDS-PAGE was done on 15% (w/v) acrylamide bis acrylamide gel. To conduct western blot, the SDS-PAGE loaded proteins were transferred to nitrocellulose membrane (Sartorius, Germany) and detected by mouse monoclonal anti-6X His tag (Antibody 18184, Abcam, Cambridge, MA) as primary antibody. Next, the Peroxidase-conjugated goat anti-mouse immunoglobulin (Dako) as secondary antibody was added. Finally, diamino benzoic acid (DAB, Amersham Bioscience, and Piscataway, NJ, USA) were applied as the chromogenic substrates.

Tat purification. E. coli BL21-DE3 cells was transformed with Recombinant Tat expression vector And induced to express protein at mid-log phase (OD600=0.5) using 1 mM IPTG. His-tagged protein was purified under native condition by applying Ni-NTA affinity chromatography column (Qiagen, USA), based on the manufacturer's instructions. Briefly, transformed E. coli BL21 (DE3) cells were gently vortexed and suspended in lysis buffer (50mM NaH2PO4. 300mM NaCl, 10mM imidazole, pH 8). To clarify the supernatant, the lysate centrifuged for 10 min at 6000×g and loaded onto a Ni-NTA column. Finally, after washing steps, the attached protein was eluted using 300mM imidazole and was loaded on SDS-PAGE to check the quality of protein purification.

Immunization procedure. A 4-year-old female Camelus dromedarius was immunized by recombinant protein as specific antigen.Onemg of purified HIV-1 Tat protein mixed with 1 ml of Frund's complete adjuvant. Four injections were done by 1 mg of antigen with 1 ml Frund's incomplete adjuvant every 14 days.

Lymphocyte isolation. After fifth injection of antigen blood sample was taken on a sterile 50mL Tube. Lymphocytes were isolated using ficole gradient sedimentation by centrifuging and lymphocyte layer separated from other layers. Purified cells were washed through PBS, count and to 106lymphocyteper cryotube was stored at -70˚C.

RNA Extraction, cDNA synthesis. RNA extraction from lymphocytes was performed by EZ-10 RNA Mini-Preps Kits from Bio Basic CANADA INC. Extracted RNA can be store at -70˚C. Extracted RNA was immediately applied for cDNA synthesis by a cDNA preparing kit from Fermentas Company and can be stored at -20˚C.

PCRs and Library production. Using cDNA as template and CALL001 and C1LL002 specific primers, the polymerase chain reaction performed by master mix solution of Qiagene Company. Produced VH fragments were observed on agarose gel electrophoresis. Products were purified by Qiagene extraction kit to apply for nested PCR. Nested PCR was performed using P38 and AGE primers to produce VHH fragments which purified by gel extraction kit from agarose gel 1%. VHH fragments were extracted using Qiagene extraction kit and applied to a ligation reaction with pHEN-4 vector to infect TG1 E. coli.

Results

Cloning and expression of Tat. After cloning of the Tat gene, enzymatic digestion was performed on pET28a vector using NcoI and XhoI restriction enzymes (Fig. 1).Different samples were gathered transfected bacteria before and after protein expression induction to perform SDS-PAGE (Figure 2).

The 14-17 kDa protein band was observed on 15% gel.

Purification of recombinant HIV-1 Tat protein. Output of Ni-NTA column after washing with elution buffer was gathered and applied for 15% SDS-PAGE.

Figure 1. Enzymatic Digestion of pET28a vector using NcoI and XhoI restriction enzymes. From left to right: 2700 bp fragment belongs to digested vector and 350 bp fragment belongs to Tat gene. Second lane belongs to undigested recombinant vector. Last lane is DNA ladder.

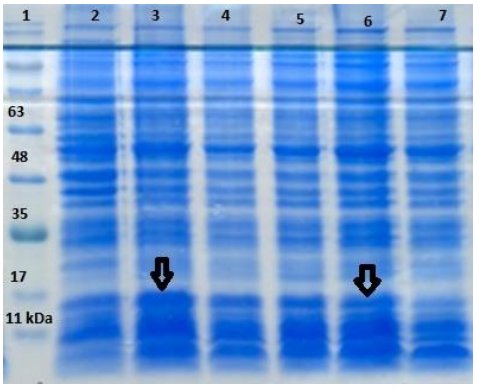

Figure 2. The samples have equal concentration of proteins on the SDS-PAGE 15%. Protein marker is the first lane. 2, 3, 4, 5, 6 and 7 are samples that were induced after 24, 4, 3, 2,1 hoursand30 minutes after induction, respectively.

Figure 3. To do western blot analysis, samples were loaded on SDS-PAGE and transferred onto nitrocellulose membrane and after adding primary and secondary antibodies was visualized by DAB.

Tat protein came out from the column after washing with elution buffer which was concluded 300mM imidazole.

PCR amplification. VH fragment amplified with CALL001 and C1LL002 primers. We were observed

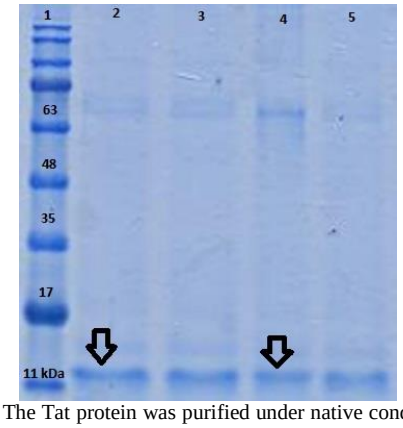

Figure 4. The Tat protein was purified under native condition using Ni-NTA column. The first lane is protein marker. Lane 2, 3, 4 and 5 are output of Ni-NTA column of 300 mM imidazole.

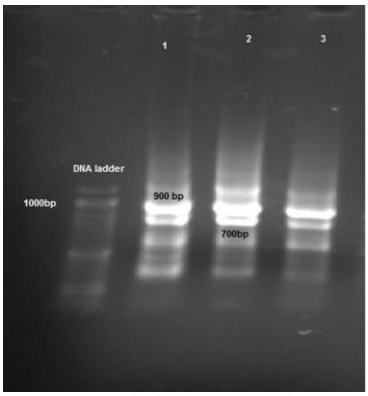

Figure 5. The result of first round of PCR products on electrophoresis of gel 1%. First lane is DNA size marker; Lane 1, 2 and 3 belongs to 900 and 700 bp fragments.

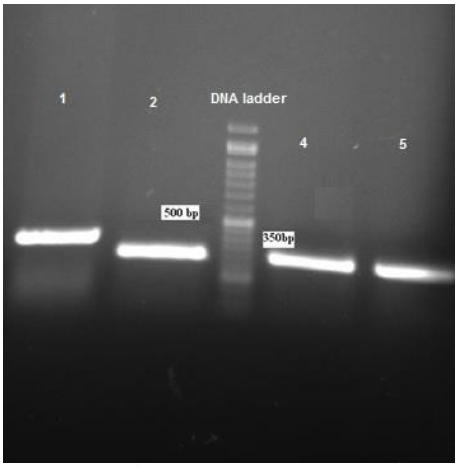

Figure 6. The result of Nested- PCR product on electrophoresis of gel 1%. Lane 1, 2, 4 and 5 have 350bp fragments which belongs to VHH gene. Lane 3 is DNA size marker.

two sharp bands of fragments upon gel about 900 and 700bp. The 700bp bands belong to VH. In order to produce VHH fragments nested-PCR was performed by P38 and AGE primers. The amplified fragment belongs to VHH gene observed 350bp.

Discussion

AIDS is acritical health problem which has affected the life of millions of people on the world.

Tat, induce virus neutralizing antibody that makes it a potential immunogenic for nanobody production. Anti-Tat monoclonal antibody (7G12) can be neutralize the variants of Tat proteins (23).

As a new strategy we want to inhibit HIV replication by 11-15 KDa nanobody because conventional antibody not able to penetrate in hidden epitopes and cannot crossed blood brain barrier (24). Tat has outstanding effect on replication and pathogenesis of HIV (18). Tat is the one of the first viral proteins that is cloned and expressed as a recombinant protein (19).

Some scientists have shown that camel nanobodies are capable to neutralize HIV-1 (25). Forsman was produced two nanobodies against the Env antigen of HIV-1 in 2008 but the antigen is not conserved in all species and doesn't has neutralization effect on broad but she implies that the VHH is able to neutralize a variety range of subtypes HIV-1 (26, 27). In 2010 Vercruysse produced an intrabody against Rev Protein of HIV-a regulatory protein that join mRNAs structure together and causes the nucleus to be let out to the cytoplasm-was disrupted the proliferation of HIV-1 by preventing the assembling of proteins by binding of the Lys-20 and Tyr-23 subunits in the N-Terminal Therefore Rev has a crucial role in virus replication (28). Our result indicates that Tat protein of HIV was cloned, expressed and purified successfully. After camel immunization and bleeding, lymphocytes isolated and RNA extracted, cDNA was synthesized and DNA amplification carried out by PCR. Then, 600 and 350 bp fragments are prepared and ligation conducted to generate a phage library. In future studies, we are going to carry out bio panning and isolate nanobody to and evaluate its therapeutic effects on HIV virus replication.

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