Original Article

Cloning of a Recombinant Plasmid Encoding Thiol-Specific Antioxidant Antigen (TSA) Gene of *Leishmania major* and Expression in the Chinese Hamster Ovary Cell Line

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Abstract

Background: **TSA (thiol-specific antioxidant antigen) is the immune-dominant antigen of *Leishmania major* and is considered to be the most promising candidate molecule for a recombinant or DNA vaccine against leishmaniasis. The aim of the present work was to express a plasmid containing the TSA gene in eukaryotic cells.**

Methods: **Genomic DNA was extracted, and the TSA gene was amplified by polymerase chain reaction (PCR). The PCR product was cloned into the pTZ57R/T vector, followed by subcloning into the eukaryotic expression vector pcDNA3 (EcoRI and HindIII sites). The recombinant plasmid was characterised by restriction digest and PCR. Eukaryotic Chinese hamster ovary cells were transfected with the plasmid containing the TSA gene. Expression of the *L. major* TSA gene was confirmed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and Western blotting.**

Results: **The plasmid containing the TSA gene was successfully expressed, as demonstrated by a band of 22.1 kDa on Western blots.**

Conclusion: **The plasmid containing the TSA gene can be expressed in a eukaryotic cell line. Thus, the recombinant plasmid may potentially be used as a DNA vaccine in animal models.**

Keywords: *CHO cells, gene expression, genetics, Leishmania major, plasmid, recombinant DNA*

Introduction

Leishmaniasis is a parasitic disease caused by several species of the genus *Leishmania*. The disease is prevalent in many parts of the world, with about 12 million total cases worldwide. As many as 1.5–2 million new cases of cutaneous leishmaniasis and 500 000 cases of visceral leishmaniasis are reported every year (1–3).

Treatment of leishmaniasis is complicated due to its toxicity, side effects, and resistance to available drugs (4). Development of new anti-*Leishmania* drugs is needed, but a *Leishmania* vaccine would offer an attractive alternative. Immunity against reinfection is acquired following cutaneous infection with *Leishmania* spp., suggesting that prophylactic immunisation is feasible (5).

In recent years, significant progress has been made in the identification of vaccine candidates capable of inducing a protective response against *Leishmania*. However, no protective, effective anti-*Leishmania* vaccine is presently available, despite several tested protocols. Thiol-specific

antioxidant antigen (TSA) has been shown to be a potentially suitable candidate molecule for a vaccine (6–9).

The Friedlin strain *L. major* genome is approximately 34 Mb and is distributed over 36 chromosomes. Its G+C content is estimated to be approximately 63%. TSA is the *L. major* recombinant protein homologue of eukaryotic thiol-specific antioxidant protein. This 22.1 kDa protein is composed of 200 amino acids and is located on chromosome 15. TSA is expressed in *L. major* promastigotes and amastigotes (10) and was chosen as a vaccine candidate because it elicits a good Th1 response in *L. major*-infected BALB/c mice. In previous studies, TSA DNA–vaccinated mice showed excellent and strong protection compared with mice vaccinated with other DNA vaccines. The TSA vaccinated mice had high titres of specific IgG1, IgG2a antibodies, high levels of IFN-γ, low levels of IL-4, and phenotypic markers of Th1 responses (1,3,11).

The aim of the present study was to construct a pcDNA3 eukaryotic expression vector containing the TSA gene of *L. major*. The ability of the construct to induce protein expression in mammalian cells was confirmed using Chinese hamster ovary (CHO) cells.

Materials and Methods

In a previous study, genomic DNA was extracted from MRHO/IR/75/ER of *L. major*, and the TSA gene (Accession number: LmjF15.1080) was amplified by PCR. The PCR product was then cloned into the pTZ57R/T vector and transformed into *Escherichia coli* (TG1 strain) (12).

Plasmid construction

To subclone TSA into the pcDNA3 plasmid, the gene was cloned with linkers to join it to the HindIII and EcoRI sites of pcDNA3 (Invitrogen, US) to produce the recombinant eukaryotic expression plasmid pcTSA. The upstream primer for the TSA gene contained a HindIII site and the ATG start codon, and the downstream primer contained an EcoRI site and the TAA stop codon. Competent *E. coli* cells (TG1 strain) were transformed with the ligation mixture by the heat shock method (13). The plasmid was then purified using a plasmid extraction kit (Bioneer, DE) according to the manufacturer's instructions and sequenced. DNA concentrations were measured by absorbance at 260 nm. The OD260/280 ratios for the purified DNA were 1.80–1.95. The plasmid recovered from the recombinant bacterial colony was sequenced by Takapou Zist Co. (IR) to confirm the presence of the TSA gene (12).

Transfection of recombinant pcTSA into eukaryotic cells

CHO cells were grown to 60%–70% confluence at 37 °C and 5% CO_2 in 35-mm wells in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, UK) containing 100 IU/mL penicillin, 100 µg/mL streptomycin, and 10% foetal calf serum (FCS). The cells were washed in serum-free medium. Transfection was then performed using a transfection kit (Genejuice Transfection Kit, Novagen, US) according to the manufacturer's instructions, and cells were incubated overnight at 37 °C in 5% CO_2. Serum-free DMEM medium was mixed with Genejuice reagent and incubated for 10 min, and then the recombinant plasmid was added and incubated for 15 min. After addition of DMEM medium with FCS, the mixture was added to cells and incubated overnight at 37 °C in 5% CO_2.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis and Western blot analysis

Transfected and untransfected cells were cultured for either 48 h or 72 h following respectively, and were harvested and lysed in sample buffer. After sonication and freezing-thawing (10 times using liquid N_2 and a 37 °C water-bath), the cells were centrifuged. The protein profile of the supernatant was resolved using 12.5% reducing sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli (14) and stained with Coomassie blue. An additional SDS–PAGE was performed for Western blotting, and proteins were transferred to nitrocellulose membranes.

Membrane strips were blocked with 1% bovine serum albumin in phosphate-buffered saline–Tween 20 solution (BSA–PBST20) overnight and sequentially incubated with *Leishmania* antibody-positive mouse serum (provided by Dr MZ Hassan, Department of Immunology, Tarbiat Modares University, Tehran, Iran) and anti–mouse IgG horseradish-peroxidase secondary antibody (Sigma, US) diluted in 1% BSA–PBST20 (1/10 000). In addition, some strips were incubated with anti-His–tagged horseradish peroxidase (1/2 000 dilution, Qiagen, US). Specific binding was shown with diaminobenzidine (DAKO, DK) (13,14).

Results

In this study, we constructed a novel plasmid containing pcTSA and transfected it into eukaryotic cells. After ligating PCR-amplified gene into the mammalian expression vector pcDNA3 and performing a restriction digest with EcoRI and HindIII, 2 bands were observed by agarose gel electrophoresis for the plasmid containing the insert, compared with 1 band for pcDNA3 digested with the same enzymes (Figure 1).

Next, SDS–PAGE and Western blot analysis were performed to confirm expression of the TSA protein. A band of 22.1 kDa was observed in the transfected cell lysates by SDS–PAGE (Figure 2). Furthermore, Western blotting using both *Leishmania* antibody-positive mouse serum and anti-His–tagged antibody revealed a band at about 22.1 kDa in cells transfected with pcTSA (Figure 3).

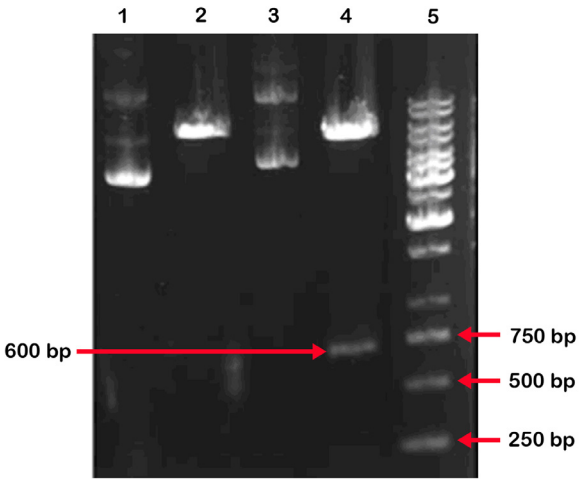

Figure 1: Electrophoresis of purified pcDNA3 and pcTSA plasmids before and after digestion with enzymes. Lanes 1 and 2: pcDNA3 (uncut and cut, i.e., digested by EcoRI and HindIII). Lanes 3 and 4: pcTSA (uncut and cut). Lane 5: O'GeneRuler 1 kb DNA ladder. Lane 1 is undigested supercoiled plasmid pcTSA. Supercoiled plasmid is denser than linear plasmid and thus migrates through the gel faster than the ladder. The 2 bands in Lane 4 correspond to the 600 bp TSA gene and the vector backbone of more than 5 000 bp.

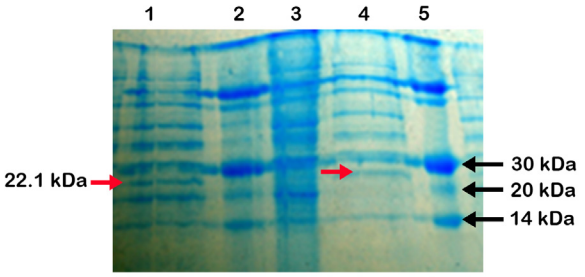

Figure 2: SDS–PAGE analysis of expressed gene product. Lane 1: CHO cells 72 h after transfection with TSA. Lane 2: Untransfected CHO cells after 72 h. Lane 3: Untransfected supernatant CHO cells after 48 h. Lane 4: CHO cells 48 h after transfection with TSA. Lane 5: Low molecular weight markers (14, 20, 30, 43, 67, and 94 kDa).

Discussion

Leishmania can cause a wide range of human diseases, ranging in severity from spontaneously healing skin lesions to fatal visceral disease. Although measures may be taken against vectors and reservoirs, and identification of new drugs is a desirable goal, particularly in view of emerging drug resistances, the development of safe and efficient vaccines remains the best hope for definitively controlling disease (6). Immunity against reinfection is acquired following cutaneous infection with *Leishmania* spp., suggesting that prophylactic immunisation is possible (5,11). *Leishmania* vaccine strategies have evolved from crude parasite preparations to defined molecules administered as recombinant proteins or DNA vaccines. DNA vaccines have some notable features compared with traditional vaccines: they are easy to produce, relatively inexpensive, homogeneous, heat stable, and believed to be safer than subunit or viral vector-based vaccines. DNA vaccines can induce strong, long-lasting and powerful humoural and cellular immunity. They also have the potential to increase immunogenicity through modifications of the vector or incorporation of adjuvant-like cytokine genes (15–17). DNA vaccines may be especially useful for protection against cutaneous leishmaniasis because the progress of naturally acquired immunity from a primary exposure to *L. major* has recently been shown to depend on both CD4+ and CD8+ T cells.

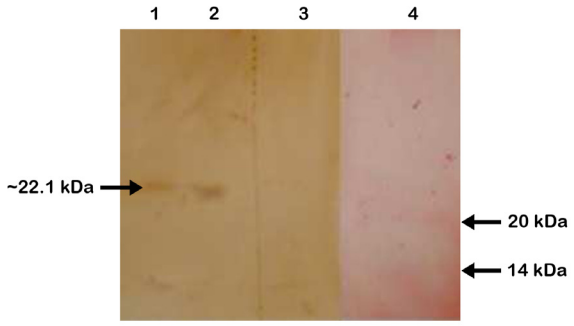

Figure 3: Western blot analysis of expressed gene product . Lane 1: Cell lysates probed with anti-His–tagged horseradish peroxidise. Lane 2: Cell lysates probed with *Leishmania* antibody-positive pooled mice sera. Lane 3: Protein extract of untransfected CHO cells probed with anti-His–tagged horseradish peroxidise. Lane 4: Low molecular weight markers (14, 20, 30, 43, 67, and 94 kDa).

In recent years, significant advances in determining the best kind of vaccine to induce an optimal immune response have been achieved. For example, antigens such as LACKp24, TSA, LmSTI1, and CPA have been tested as candidate DNA vaccines for *Leishmania*. All of these antigens were able to elicit relatively protective effects, but none could confer complete protection.

Recently, type I, II, and III cysteine proteinases have become attractive candidates as vaccine antigens against visceral leishmaniasis (7,11,18–21). In an attempt to develop a DNA vaccine against cutaneous leishmaniasis, we focused on the gene encoding TSA because previous studies have shown that immunisation with TSA peptides, proteins, or DNA can elicit a broad range of humoural and cellular immune responses in animals infected with *L. major*. *Leishmania* TSA protein is known to be antigenic in both murine and human systems and is constitutively expressed in both promastigote and amastigote life stages. Moreover, this antigen is abundant and homogeneously distributed on the surface of both extracellular and intracellular promastigotes and amastigotes (10).

Immunisation of BALB/c mice with TSA DNA vaccine results in high levels of protective immunity (humoural and cellular), induces cytotoxic lympocytes activity, and robust protection, and stimulates high titres of specific IgG1 and IgG2a antibodies. It also induces strong IFN-γ production, with low levels of IL-4 and phenotypic markers of Th1 response (1,3,11). Both LmSTI1 and TSA antigens confer outstanding protection in both murine and nonhuman primate models of human cutaneous leishmaniasis. The recombinant TSA protein, along with IL-12 , has also been shown to induce good protection in murine models. As it induces a CD8 response and specific immunity, which can suppress parasite numbers, TSA DNA may be considered the vaccine of choice (2).

Thus, the production of TSA protein is a critical basis for further research into and development of a sophisticated, effective vaccine against leishmaniasis.

Conclusion

In this study, we successfully expressed recombinant plasmid containing TSA gene of *L. major* in eukaryotic cells, further demonstrating its potential to be used as a DNA vaccine in animal models.

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Authors' Contributions

Conception and design, obtaining of funding, provision of study materials, collection, assembly, analysis, and interpretation of the data, statistical expertise, drafting of the article: FG, FT
Critical revision and final approval of the article: FG, FT, ZS, AD, MZH, MM

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