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Expression of a Chimeric Protein Containing the Catalytic Domain of Shiga-Like Toxin and Human Granulocyte Macrophage Colony-Stimulating Factor (hGM-CSF) in *Escherichia coli* and Its Recognition by Reciprocal Antibodies

Mehryar Habibi Roudkenari[1], Saeid Bouzari[*1], Mana Oloomi[1], Anis Jafari[1], Nader Shahrokhi[1] and Mohammad Ali Shokrgozar[2]

[1]*Molecular Biology Unit and* [2]*National Cell Bank, the Pasteur Institute of Iran, Tehran, Iran*

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ABSTRACT

Fusion of two genes at DNA level produces a single protein, known as a chimeric protein. Immunotoxins are chimeric proteins composed of specific cell targeting and cell killing moieties. Bacterial or plant toxins are commonly used as the killing moieties of the chimeric immunotoxins. In this investigation, the catalytic domain of Shiga-like toxin (A1) was fused to human granulocyte macrophage colony stimulating factor (hGM-CSF) gene and the fused gene was then expressed using an expression vector containing arabinose promoter. The protein thus obtained could be recognized by two different ELISA system designed for detection of hGM-CSF and Shiga-toxin and reconfirmed by Western-blot. The recognition of the chimeric protein by specific antibodies could be indicative of the proper form of the protein, which justifies further steps to be taken to evaluate the potential effects of the chimeric protein. *Iran. Biomed. J. 9 (4): 143-148, 2005*

Keywords: Expression, Chimeric protein, Shiga-toxin, Human granulocyte macrophage colony stimulating factor (hGM-CSF), *Escherichia coli*

INTRODUCTION

It was Paul Ehrlich who suggested over 100 years ago to bind medications to specific carriers that would carry the drug to the infected cells. These carriers were designated "Magic Bullets" which, theoretically, could cure all diseases, providing their pathology were known. With progress in molecular biology, a variety of chimeric proteins that fit Ehrlich's definition have been constructed by fusing two genes at DNA level to produce a single protein, known as chimeric protein. These molecules are composed of a cell targeting and cell killing moieties that enable them to recognize and destroy cells overexpressing specific receptors involved in a variety of human diseases [1].

Bacterial and plant toxins have been attached chemically or genetically to monoclonal antibodies and polypeptide hormones to target their cytotoxicity toward euokaryotic cells [1]. In most cases, the bacterial toxins diphtheria toxin (DT) and Pseudomonas exotoxin A have been used as the toxic elements which act by inhibiting protein synthesis [2]. Another such toxin is Shiga-toxin/Shiga-like toxins, belonging to the AB family [2] which are composed of A subunit (catalytic domain) and B subunit (binding domain) [3]. The A subunit is composed of A1 and A2 fragments. The A1 fragment inhibits protein synthesis after release in the cytosol by removing one adenine from 28S rRNA of 60S ribosomal subunit [4].

Human granulocyte macrophage colony-stimulating factor (hGM-CSF) is a 127-amino acid long cytokine which is responsible for growth, differentiation and functional enhancement of granulocytes and macrophages [5]. The GM-CSF receptor (GM-CSFR) is expressed as a heteromeric complex containing an α and a β subunit [6]. Such receptors with a narrow tissue distribution and a high affinity are promising targets for selectively delivering toxins to malignant cells [7].

*Corresponding Author; Tel. (+98-21) 6695 3311-20; Fax: (+98-21) 6646 5132; E-mail: saeidbouzari@yahoo.com

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Here we report the construction, expression and characterization of a fusion protein termed A1-GM-CSF chimeric protein in which the cell-surface binding domain of Shiga-toxin was replaced with mature hGM-CSF. The fusion protein was produced in *E. coli* and its ability to be detected with respective antibodies was assessed.

MATERIALS AND METHODS

Plasmids and bacterial strains. Plasmids *p*ET28a (Novagen, USA) and *p*BAD/gIII A (Invitrogen, USA) were used for construction and expression. Bacterial strains *E. coli* BL21 (DE3) plysS (Novagen, USA) and Top10 (Invitrogen, USA) were used for expression of the constructs. *p*UC18 was used for initial cloning and sequencing. *p*BAD/gIII expression vector provides the opportunity to clone the desired insert as a fusion protein with two tags: C-terminal *myc* epitope and polyhistidine region. These tags facilitate detection of the expressed protein with anti-*myc* and anti-his antibodies and also purification of the protein using the metal-binding site for affinity purification of the recombinant protein .

DNA extraction. Genomic DNA was extracted from *E. coli* strain O157:H7 by standard method [8] and used as PCR template for amplification of catalytic domain of Shiga-toxin.

Table 1. list of primers.

No.	Primer name	Sequence
1	A1-F	5'-cat gcc atg gaa ttt acc tta gac ttc tc g-3'
2	A1-R	5'-tta gct cga gat cag atg cca ttc tg-3'
3	A1GM-CSF-R	5'-**ggc ggg tgc** atc aga tgc cat tct ggc-3'
4	GM-CSFA1-F	5'-**atg gca tct gat** gca ccc gcc cgc tcg ccc-3'
5	GMCSF-R	5'-cca ctc gag ctc ctg gac tgg ctc-3'

Amplification of catalytic domain of shiga-toxin and hGM-CSF and construction of the fused gene. Catalytic domain of Shiga-like toxin was amplified using specific primers no. 1 and 2 (Table 1) containing *Nco*I and *Xho*I sites (underlined). All PCR reactions in aliquots of 50 µl contained 5 µl of 10X PCR buffer, with $MgSO_4$, 200 mM (each) deoxynucleoside triphosphates (dNTP), 15 pmol of each primer and 200-500 ng of template DNA. PCR was carried out in Eppendorf Thermo Cycler using *Pfu* (Fermentas, Lithounia) with initial denaturation at 95°C for 5 min followed by 1 min at 94°C, 45 s at 60°C and 45 s at 72°C for 30 cycles. PCR product was visualized by UV and fragment of expected size was eluted by gel purification kit (Roche Diagnostic, Germany) and digested with respective restriction enzymes. The digested product was cloned and sequenced by a commercial facility (MWG, Germany). A previously cloned GM-CSF gene in *p*UC18 [9] was used as a template for PCR using primers no. 4 and 5 (Table 1). A1 region was amplified using primers no.1 and 3 (Table 1). Primer no.3 is composed of nucleotides complementary to both 3'-end of A1 domain of Shiga-like toxin and 5'-end of GM-CSF (in bold). Similarly GM-CSF was amplified using primers no. 4 and 5. Primer no. 4 contains a region complementtary to 3'-end of the A1 catalytic domain in addition to bases complementary to GM-CSF (Table 1). Equal mM of the PCR products thus obtained were used as template with primers no.1 and 5 for amplification of the fused gene using a two-step programme as follows:

After denaturation at 94°C for 3 min, mixed PCR products were allowed to anneal at 45°C for 2 min. To this mixture, was then added the PCR reagents (5 µl of 10X PCR buffer containing $MgSO_4$, 200 µM (each) of dNTP, 15 pmol of each primer and 2.5U of *Pfu* enzyme) and amplification was allowed to continue for 30 cycles at 94°C for 1min, 65°C for 45s and 72°C for 40s, a final extension of 7 min at 72°C was included as the final step. All the primers used for amplification of various fragments used in this study were synthesized by MGW, Germany.

PCR product of expected size was gel purified using Gel Purification Kit (Roche Diagnostic, Germany). Construction of the fused gene encoding A1-GM-CSF is summarized in Figure 1. The fused gene (A1-GM-CSF) was digested with *Nco*I and *Xho*I enzymes incorporated at 5' and 3'-end of the primers, respectively. Expression vectors *p*ET28a and *p*BAD/gIII A were similarly digested using the same enzymes and gel-purified vectors and insert were ligated. The ligation product was transformed into competent and *E. coli* BL21 (DE3) plysS and Top10, respectively and transformed cells were selected on Lauria Bertani plates containing ampicillin. The clones thus selected were induced by different concentration of isopropyl-β-D-thiogalactopyranoside or arabinose as instructed by the manufacturers. The induction was carried out when culture had reached the OD of 0.5 at 600 nm and a further incubation at 37°C for 4 h and 6 h.

Prior to induction, a selected clone containing the insert was sequenced by a commercial facility (MWG, Germany).

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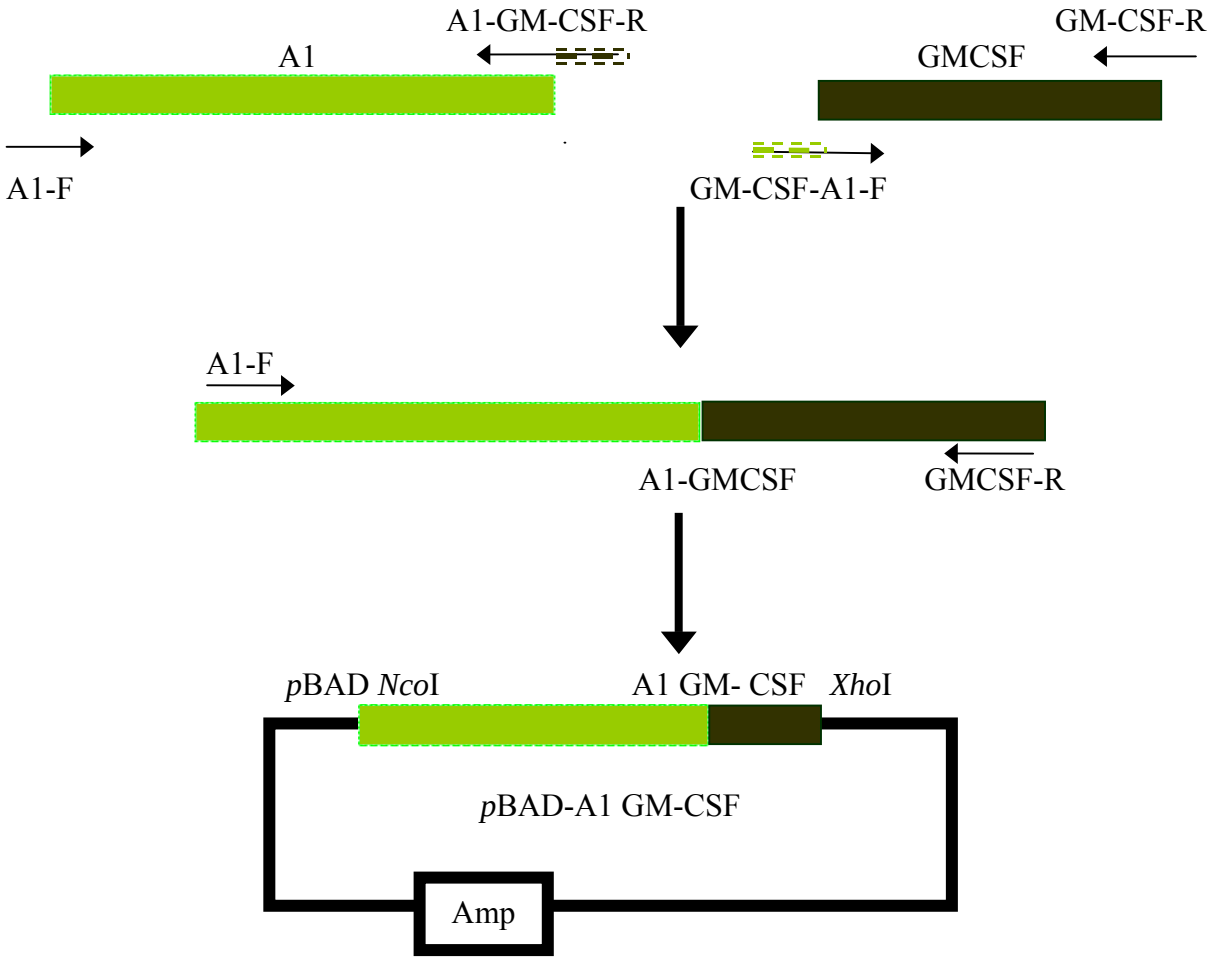


Fig. 1. Schematic presentation of fusion of A1 and hGM-CSF genes (overlapping segments in the primers used are shaded).

Detection of expressed chimeric protein. Expressed protein was detected by running the samples heated in 1X SDS-PAGE sample buffer at 70°C for 5 min on 15% gel and stained with Coomassie blue. The proteins were also blotted onto nitrocellulose paper (Hi-bond Amersham Biosciences, USA) and blocked with a solution containing 0.1% skimmed milk and 0.1% Tween 20. Blocked membranes were washed with PBS-0.05% Tween 20 and incubated with horseradish peroxidase-conjugated *myc* antibody (Invitrogen, USA) at room temperature for 2 h. Membranes were then washed 3 times with PBS containing 0.05% Tween 20 and developed using 3,3`-diaminibenzidine tetrahydrochloride, fermentas as substrate. Antigenicity of the expressed protein was assessed by Western-blot using hGM-CSF antibody (R & D, USA) with 0.7 µg/ml peroxidase-conjugated rabbit anti-goat IgG (Dako, Denmark) as secondary antibody at room temperature for 1 h.

Membranes were developed with 5µl of 3% H_2O_2 (Sigma, USA). The expressed fusion protein was also checked using two commercially available ELISA kits designed for detection of hGM-CSF (R & D system, USA) and Shiga-toxin (RIDA-SCREEN®, r-Biopherm). Gels were scanned using Bio-Rad MultiAnalyst™ /PC version1.1.

RESULTS

Amplification of fused gene. Schematic representation of amplification and assembly of A1 and GM-CS, given in Figures 1 and 2, shows amplification of each domain and their assembled product. The nucleotide sequences of fused genes and their junction were confirmed by sequencing. The fused gene sequence was searched for homology by BLAST analysis, and it confirmed the identity of the two genes fused together.

Construction and expression of A1-GM-CSF. The expression vector containing chimeric A1-GM-CSF gene was constructed as described. The

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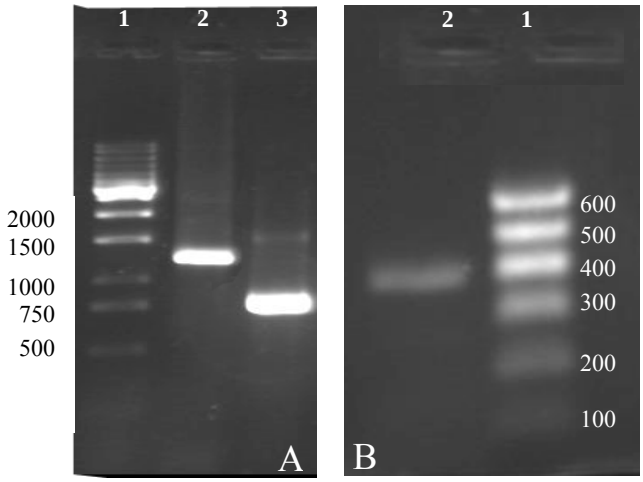


Fig. 2. Amplicons representing the catalytic domain of Shiga-like toxin (A1) and fused A1-GM-CSF. **(A)** Lane 1, MWM; Lane 2, fused A1-GM-CSF genes; Lane 3, A1. **(B)** Lane 1, MWM; Lane 2, GM-CSF.

resulting fusion protein, A1-GM-CSF is composed of amino-terminal amino acids 1-255 of A1 catalytic domain of Shiga-like toxin, fused to amino acids 1-127 of hGM-CSF through an overlap region of the two genes created by primer design. The *p*BAD/gIII can be used to add a C-terminal *myc* epitope and a polyhistidine tag, facilitating detection and purification of the expressed protein. In this study, hGM-CSF fragment was used without a stop codon, therefore, the hybrid gene was expressed containing both C-terminal *myc* epitope and polyhistidine region. The calculated molecular mass of A1-GM-CSF was approximately 43 kDa.

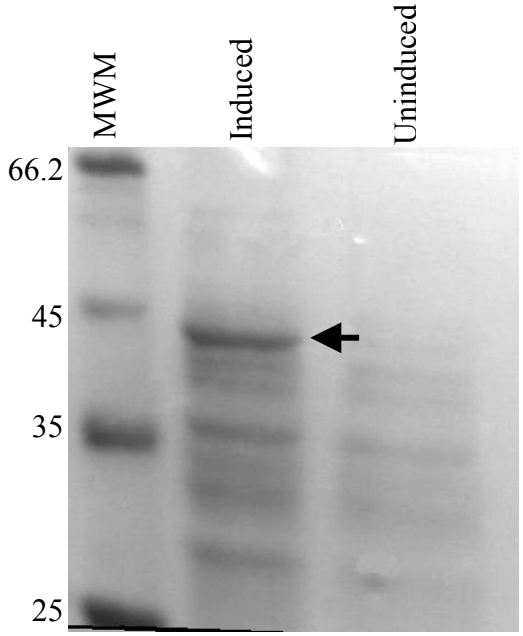


Fig. 3. DS-PAGE showing expression of A1-GM-CSF after induction with 0.02% concentration of arabinose.

Top10 cells transformed with *p*BAD/gIII-A1-GM-CSF were treated with different concentration of arabinose as instructed by the manufacturer. The induction was carried out for 4 h, and the cells were collected and processed as instructed.

Aliquots were dissolved in SDS-PAGE sample buffer (whole-cell extract) and analyzed by electrophoresis. A protein of approximately 43 kDa was induced (Fig. 3). The optimization of protein expression was attempted in different range of arabinose (0.002-2%) concentration and induction period (data not shown). The best result was obtained at 0.02% arabinose and 4 h incubation.

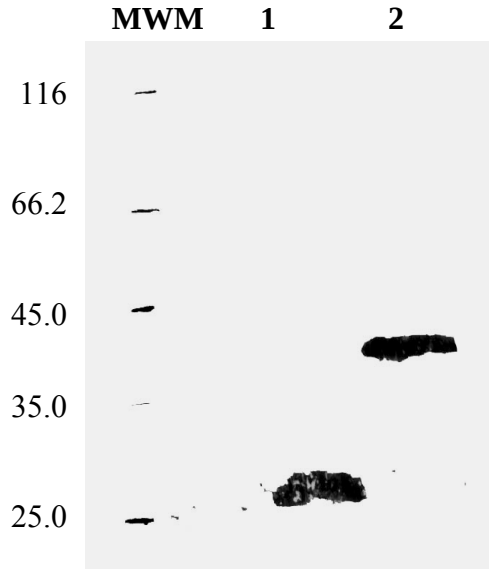


Fig. 4. Western-blot analysis of A1-GM-CSF using anti-*myc*. Lane 1, Calmodulin; Lane 2, A1-GM-CSF.

Detection of expressed chimeric protein. Western-blot analysis was performed for the chimeric protein using anti-*myc* antibody included in the kit (Fig. 4). *p*BAD/gIII A containing calmodulin gene was also induced as positive control as recommended for optimization of arabinose concentration, period of induction, mode of extraction and antibody detection in Western-blot technique.

The chimeric protein A1-GM-CSF was detected using *myc* antibody and calmodulin as positive control to check the functionality of the antibody. The antigenicity of the chimeric protein was confirmed by Western-blotting using anti-GM-CSF antibody (Fig. 5).

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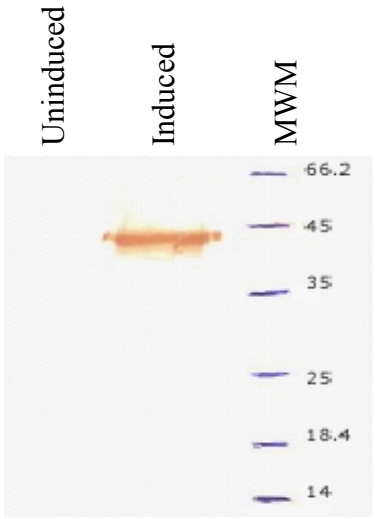


Fig. 5. Western-blot analysis of A1-GM-CSF using anti-hGM-CSF.

DISCUSSION

The chimeric protein containing catalytic domain of Shiga-like toxin and hGM-CSF was constructed. In native form, the receptor-binding domain of Shiga-toxin is located at the 3'-end of the gene and the carboxy terminus of the protein [3]. Therefore, GM-CSF was placed at the carboxy terminus of Shiga-toxin in place of the binding domain. In similar studies, a chimeric toxin was constructed in which GM-CSF was fused to the carboxy terminus of DT [10] and it was shown that it is toxic to human acute myeloid leukemia (cells bearing GM-CSF receptors) [11]. Similarly, A subunit of Shiga-toxin was genetically fused to a 165-amino acid polypeptide derived from CD4, the cellular receptor for human immunodeficiency virus type1 (HIV-1) This strategy eliminated the STX receptor binding subunit and directed the hybrid toxin to cells expressing the HIV-1 surface glycoprotein gp120 [12]. In the present study, attempts were made to express the chimeric protein A1-GM-CSF in *p*ET28a, the vector with strong T7 promoter, despite all attempts (data not shown), the expression was not successful. It has been demonstrated that over-expression of SXT enzymatic (A1) polypeptide which lacks a signal sequence caused a reduced rate of growth of its *E. coli* host due to its inhibitory effect on prokaryotic translation system [13]. Our failure to express A1-GM-CSF in *p*ET28a, that expresses the protein in cytoplasmic form, could be explained based on the Skinner *et al.* [13] observation. It was also shown that fusion toxin DT::CT, despite its cytotoxicity was not suitable for clinical trial due to its low expression level, requirements for solubilization and subsequent refolding and concerns about bacterial endotoxin contamination. These problems prompted investigation into the utility of baculovirus/insect cell expression system for the production of therapeutic fusion toxins [14]. In this study, *p*BAD/gIII expression vector, which is a tightly regulated system based on arabinose operon was employed, and it is designed to express the desired protein in preplasmic space. Expression of the heterologous recombinant proteins in preplasmic space abolishes the need for denaturation/ renaturation cycle which is required when proteins are produced in the form of inclusion bodies. Our data showed that *p*BAD/gIII is an efficient system for expression of this fusion toxin. Previous attempts to express A subunit have demonstrated that the level of expression was low and immunoprecipitation was needed to detect the protein [15, 16]. However, using *p*BAD/gIII resulted in an expression level which was in a detectable range in SDS-PAGE and this demonstrates the efficiency of the expression vector and optimization of the expression conditions. Moreover, the expressed protein was confirmed by Western-blot analysis using anti-GM-CSF antibody and anti-Shiga-toxin, that not only confirmed the presence of the fusion protein, but also its antigenicity, that could be recognized by reciprocal antibodies. These initial findings warrant further steps to determinate the full potency of the fusion toxin and its cytotoxic effect on the GM-CSF receptor-bearing cells. Furthermore, attempts will be made to construct the fusion toxin without *myc* epitope which would enable us to assess the effect (if any) of *myc* epitope on receptor binding ability of hGM-CSF moiety.

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