

***In vivo* Characterization of Fusion Protein Comprising of A1 Subunit of Shiga Toxin and Human GM-CSF: Assessment of Its Immunogenicity and Toxicity**

Mana Oloomi, Saeid Bouzari* and Elaheh Shariati

Molecular Biology Unit, Pasteur Institute of Iran, Pasteur Ave. Tehran 13164, Iran

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ABSTRACT

Background: Most cancer cells become resistant to anti-cancer agents. In the last few years, a new approach for targeted therapy of human cancer has been developed using immunotoxins which comprise both the cell targeting and the cell killing moieties. **Methods:** In the present study, the recombinant Shiga toxin A1 subunit fused to human granulocyte-macrophage colony stimulating factor (A1-GM-CSF), previously produced in *E. coli*, was further characterized. **Results:** The recombinant protein could cause 50% cytotoxicity and induced apoptosis in cells bearing GM-CSF receptors. The non-specific toxicity of the fusion protein was assessed in C57BL/6 and BALB/c mice. No mortality was observed in either group of mice, with different concentration of fusion protein. **Conclusion:** The lymphocyte proliferation assay, induction of specific IgG response and a mixed (Th1/Th2) response were observed only in BALB/c mice. The mixed response in BALB/c mice (Th1/Th2) could be explained on the basis of the two components of the fusion protein i.e. A1 and GM-CSF. *Iran. Biomed. J. 14 (4): 136-141, 2010*

Keywords: Cell line, Fusion protein, Toxicity

INTRODUCTION

In the past several years, immunotoxins have been used to target cancer cells [1]. Nowadays, immunotoxins are designed to contain only the elements required to recognize and kill the tumor cells. This is accomplished by replacing the cell-binding domain of the toxin with portion of the antibody or the growth factor. Growth factors and cytokines as well as immunologic proteins smaller than monoclonal antibodies have also been chemically conjugated or genetically fused to protein toxins [2]. DT-GM-CSF (Diphtheria toxin fused to granulocyte-macrophage colony stimulating factor) is a recombinant toxin that targets the GM-CSF receptor that is present on acute myeloid leukemia cells [3]. The preclinical experiments using recombinant immunotoxins have suggested that these molecules are promising agents for cancer therapy. However, these molecules proved to be difficult to treat cancer due to their non-specific toxicity [4]. Toxicity associated with immunotoxins

can be either non-specific or targeted [5]. The high concentration of cytotoxicity can cause non-specific toxicity [6]. The second type of toxicity can cause targeting the toxin to normal tissues that contain the same target antigen as the cancer cell [2]. The A1-GM-CSF (Shiga toxin A1 subunit fused to GM-CSF) fusion protein, constructed in our previous study [7], was further assessed for its toxicity in *in vivo* assay. Therefore, its toxicity was determined in C57BL/6 and BALB/c mice. In addition, the modulatory effect of A1-GM-CSF on immune response of injected mice was also determined.

MATERIALS AND METHODS

Purification of hybrid protein. Medium containing constructed clone was induced by optimized concentration of L-arabinose. After centrifugation, the pellet was used for purification of the expressed protein. The bacterial cells from the pellet were suspended in 0.1% polymyxin B in PBS

*Corresponding Author; Tel. (+98-21) 6695 3311; Fax: (+98-21) 6649 2619; E-mail: saeidbouzari@yahoo.com

(pH 7.4) and incubated at 37°C for 60 min. For total protein estimation of periplasmic extract, the preparation thus obtained was applied to EndoTrap column (Profos, Germany). The expressed protein was eluted by Electro-Eluter (Bio-Rad, Life Science Group, USA) and the purified protein concentration was estimated by absorbance at 280 nm with bovine serum albumin as the standard. For determination of A1-GM-CSF concentration, the specific ELISA kit for GM-CSF (R & D Systems, Inc., USA) was used.

Accession number. A1-GM-CSF constructed gene under study was sequenced, and the sequence obtained was deposited into GenBank under accession number EF032873.

In vitro cytotoxicity of A1-GM-CSF. Cytotoxicity of the periplasmic extracts of A1-GM-CSF was checked on K562 cell (National Cell Bank, Pasteur Institute of Iran). Cells (10^5 per well) were grown in 6-well microtiter plates (Nunc, Germany) in RPMI medium (pH 7.4, supplemented with 10% heat inactivated fetal bovine serum, 100 units/ml penicillin-streptomycin) in a 5% CO₂ atmosphere at 37°C for 24 h. Briefly, serial 10-fold dilutions (100 μ l) of the periplasmic sterilized sample solution were incubated with 10^5 cells overnight and observed microscopically. An average percentage of cell death was calculated from three separate experiments. On the other hand, neutralization of the cytotoxicity was performed by incubation of antibody raised against A1-GM-CSF in rabbits with hybrid protein to inhibit the toxicity. Serum from the immunized rabbit was mixed with different dilutions of A1-GM-CSF and incubated at 37°C for 30 min. The mixture was then added to the cells (1.2×10^5 /ml) in a 96-well micro titer plate incubated at 37°C overnight. The cell viability was measured by neutral red assay [8]. Shortly, after 3 h incubation with neutral red solution, viable cells were fixed with 1% CaCl₂ in 0.5% formaldehyde for 3-5 min. Thereafter, washing cells were lysed with 1% acetic acid in 50% ethanol. The absorbance was measured by an ELISA reader (Awareness Technology Inc., USA) in 540 nm. Induction of apoptosis by the recombinant protein was measured by Cellular DNA Fragmentation ELISA kit from Roche Diagnostics GmbH, Germany.

Animals. Two groups of animals, C57BL/6 and BALB/c mice (Female, aged 4-5 weeks), were obtained from the Animal Facility of Pasteur Institute of Iran. The animals used in this study were

housed in standard Plexiglas cages with free access to food (standard laboratory rodent's chow) and water. The animal house temperature was maintained at $23 \pm 3^\circ\text{C}$ with a 12 h light/dark cycle (light on from 6 a.m.). All animal experiments were carried out in accordance with the European Communities of Council Directive of 24 November 1986 (86/609/EEC) in such a way to minimize the number of animals and their suffering. Each animal was tested once. A New Zealand white rabbit was injected subcutaneously with 500 μ g (0.1 ml) purified A1-GM-CSF. This hybrid protein was mixed with an equal volume of Freund's Complete Adjuvant (Sigma Chemical Co., St. Louis, Mo., USA). The immunization was repeated at 4 and 6 weeks after the first injection with Freund's Incomplete Adjuvant. All injections were given subcutaneously at multiple sites in the dorsal region. The rabbit was bled 1 week after the third injection and serum was collected.

Toxicity study. Five groups of C57BL/6 and BALB/c mice (six in each) were injected i.p. with 10, 20, 50, 100 and 200 μ g/day of periplasmic extract containing A1-GM-CSF for 5 consecutive days. The periplasmic extract was checked by Western-blot analysis using Anti-A (polyclonal antibodies against A subunit of Shiga toxin) and Anti-GM-CSF (R&D Systems, Inc., USA) antibodies. As a control periplasmic extract of the construct without the gene was used for injection to mice. Animals were then observed twice daily for mortality until the day 26 (21 days after the last injection of the extract), according to the method described by Hall *et al.* [6].

Detection of mouse IgG response to A1-GM-CSF. On day 26 (21 days after the last injection of A1-GM-CSF), retro-orbital blood samples (50 μ l) were drawn. The serum was separated and frozen until assay. The 200 μ l per well of purified A1-GM-CSF (10 μ g/ml) was plated on 96-well ELISA plates (Nunc, Germany) at 4°C overnight. The plate was then washed four times with PBS plus 0.1% Tween-20 followed by the addition of 200 μ l of PBS/skim milk at room temperature for 1 h. Three 10-fold dilutions of the serum were performed, and 200 μ l of each sample was added per well at room temperature for 2 h. The plate was then washed four times and 100 μ l of a 1:2000 dilution of goat anti-mouse IgG peroxidase (Sigma, USA) was added at room temperature for 1 h. Then, the plate was washed four times, and 50 μ l tetramethyl benzidine was added.

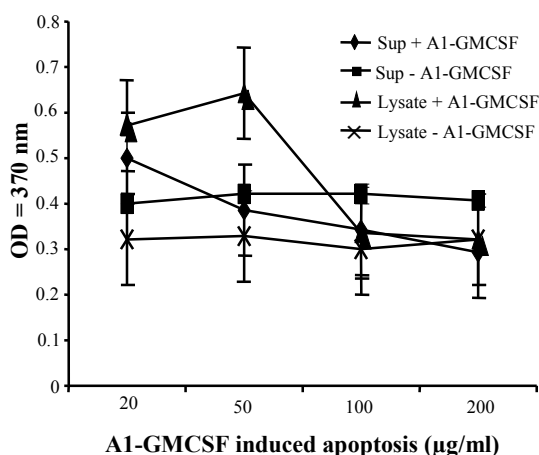


Fig. 1. Cellular DNA fragmentation assay. Cellular DNA was labeled with BrdU (5'-Bromo-2'-deoxy-Uridine). It is present in supernatant and lysate of cells and detected by anti-BrdU antibody. The A1-GM-CSF hybrid protein (20, 50, 100 and 200 µg/ml) was measured for its apoptotic cell death by DNA fragmentation in the cell lysate. Sup-A1-GM-CSF supernatant of cells not treated with A1-GM-CSF (control); Lysate-A1-GM-CSF-Lysate of cells not treated with A1-GM-CSF (Control); Sup + A1-GM-CSF-supernatant of cells treated with A1-GM-CSF; Lysate + A1-GM-CSF-Lysate of cells treated with A1-GM-CSF.

The plate was read at 450 nm after 20 min. Western-blotting was performed by commercial antibodies: anti-myc, Invitrogen Life Technologies, USA, anti-A, polyclonal antibodies against A subunit of Shiga toxin and anti-GM-CSF (R&D Systems, Inc., USA) for A1-GM-CSF characterization. The primary antibody used throughout this study was serum at a dilution of 1:10,000. The secondary antibody was Goat anti-rabbit conjugated horseradish peroxidase (Sigma, USA) and used at a dilution of 1:1,500. 3, 3'-diaminobenzidine tetrahydrochloride (Sigma, USA) was used as a substrate for detection.

Lymphocyte proliferation assay. After 26 days of the observation, the injected mice were sacrificed three weeks after the last immunization. Spleens of three mice were separated and cultured at 2×10^6 viable cells per ml in 2 ml volumes in 24-well plates. Cultures were established in the presence of A1-GM-CSF (20 µg/ml) and cell proliferation was assessed [9]. After incubation at 37°C in 5% CO₂ humidified atmosphere, cells were pulsed during the culture with 0.5 µCi [³H] thymidine (Amersham, UK), incubated for 18 h and then harvested. The thymidine incorporation was determined by liquid scintillation using a β counter (Pharmacia, USA). Data are expressed as stimulation index representing the mean ratio of counts per minute of the stimulated

culture to the mean counts per minutes of the control culture (medium). Supernatant of the above cultures was pooled and stored at -80°C for cytokine assay after 72 h incubation. IFN-γ and IL-4 production were detected by mouse IFN-γ and IL-4 Module Set (Bender MedSystems, USA) according to manufacturer's instructions. All tests were performed in triplicate for three mice.

Statistical analysis. Data were subjected to ANOVA and Student's *t*-test for statistical analysis, and a *P* value of <0.05 was considered to be significant.

RESULTS

Characterization of A1-GM-CSF. The characterized gene was deposited into GenBank under accession number EF032873. The protein thus obtained [7] was used for further studies.

In vitro study. In order to determine the cytotoxicity of the fusion protein before injection to the mice, the ability of the purified protein was assessed on K562 cell Line; the LD50 for hybrid toxin was 100 µg/ml after overnight incubation. The protein was found to be cytotoxic; its effect was neutralized by anti-A1-GM-CSF raised in rabbit, and 12% cytotoxicity was shown after inhibition. The mechanism of cytotoxicity was shown to be due to apoptosis occurred by 50 µg/ml of the hybrid protein (Fig. 1). Cellular DNA fragmentation by ELISA kit was determined and apoptotic cell death was assessed by detection of DNA fragments in the cell lysate of cells treated with hybrid protein.

In vivo toxicity of A1-GM-CSF. Initially, C57BL/6 mice were used. The mice received 10, 20, 50, 100 and 200 µg/day of periplasmic extract containing A1-GM-CSF for 5 consecutive days. They survived like the control group which received PBS. We extended the amount of hybrid toxin to 200 µg/day, and still all mice survived. Since no death was observed in C57BL/6 mice, the BALB/c mice were included in the study. Since BALB/c mice are sensitive animal, the toxicity of the A1-GM-CSF was examined and still no death occurred. Sera from each group (6 mice) were pooled, and antibody response was observed by Western-blotting in the BALB/c group who received 200 µg/day of hybrid toxin (Fig. 2A).

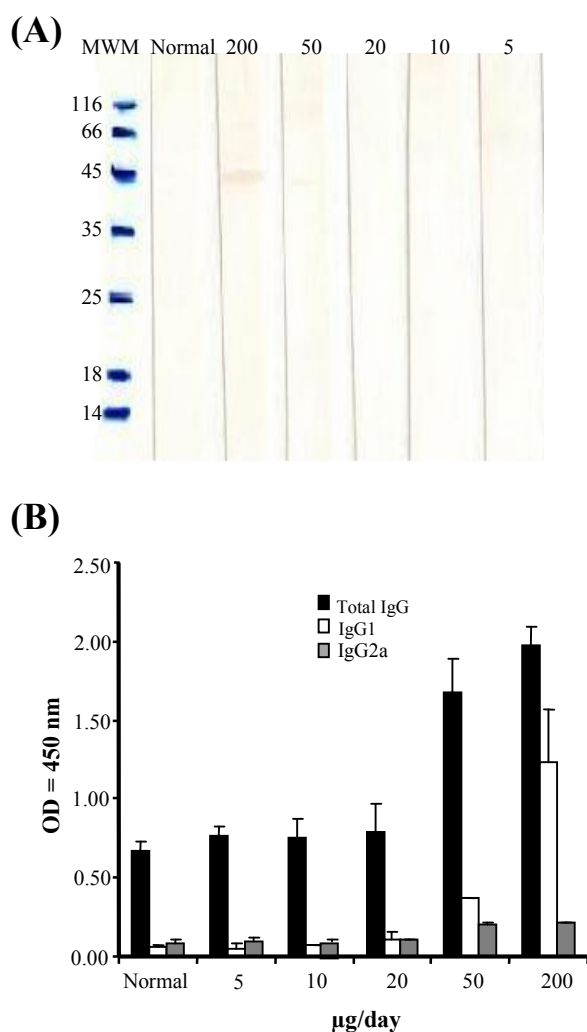


Fig. 2. Antibody response. Sera from each group (6 mice) receiving 5, 10, 20, 50 and 200 µg/day of A1-GM-CSF were pooled, and antibody response was checked in BALB/c mice by Western-blotting (A); it was shown that receiving 50 and 200 µg/day of hybrid protein induced significant total IgG, IgG1, and IgG2a immune response ($P < 0.05$) (B).

Detection of mouse IgG response to A1-GM-CSF. IgG, IgG1 and IgG2a production was also assessed by ELISA assay in each group. The sera of both groups were collected and checked for A1-GM-CSF specific total IgG production. In the case of C57BL/6 mice, no induction of the immune system was observed, but in the BALB/c mice group, induction of the immune system was just noticed with high concentration of A1-GM-CSF (200 µg/day). It was shown that receiving 50 µg/day of hybrid protein induced significant total IgG and IgG1 and IgG2a immune response ($P < 0.05$) as indicated in Figure 3B. Induction of the immune system in BALB/c mice with high dose of A1-GM-CSF was also observed in Western-blot analysis, in which the serum of mice injected with 200 µg/day of

A1-GM-CSF and reacted with A1-GM-CSF protein separated on SDS-PAGE (Fig. 2A).

Lymphocyte proliferation and cytokine production. The capacity of splenocytes to proliferate in response to A1-GM-CSF was investigated. Mice that received A1-GM-CSF displayed proliferation that was not observed in the control mice. Similarly, only spleen cell proliferation from BALB/c mice that received 200 µg/day of A1-GM-CSF was observed, but not C57BL/6 mice. The stimulation index of splenocytes with A1-GM-CSF antigen was significantly higher ($P < 0.05$) compared to control groups in BALB/c mice receiving PBS (Fig. 3). The level of IFN-γ and IL-4 production was analyzed in the supernatant of spleen cells from all groups (Fig. 3). Significant levels of IL-4 and IFN-γ production were observed during this time period while control groups produced no detectable IL-4 and IFN-γ. It was shown that the production of IL-4 was higher than that of IFN-γ in BALB/c mice receiving 200 µg/day of A1-GM-CSF (Fig. 3). The mixed response in BALB/c mice (Th1/Th2) could be explained on the basis of two components of the fusion protein i.e. A1 and GM-CSF.

DISCUSSION

Targeted toxins consisting of tumor-selective ligands coupled to polypeptide toxins represent a new class of cancer therapeutics that kills malignant cells by inactivating cytosolic protein synthesis and inducing apoptosis [10]. When the targeting moiety is a cytokine or growth factor, the molecule is referred to as a fusion protein toxin [10].

In some instances, the targeted toxin receptor is present on normal tissues, and side effects have occurred [11]. In a study conducted by Hall *et al.* [6], the DT-GM-CSF exhibited non-specific toxicity in C57BL/6 mice (10 µg/day). In our study, a much higher concentration of the protein was not found to be lethal for the C57BL/6 mice. Since GM-CSF receptor is present on mature monocytes, macrophages and neutrophils, early dose-limiting toxicity for DT-GM-CSF and cytokine release or systemic inflammatory response syndrome, were also observed [12-14]. On the other hand, no induction of the immune system, no proliferation and no cytokine production was detected in A1-GM-CSF-injected C57BL/6 mice. Based on the data obtained from animal experiments, it could be

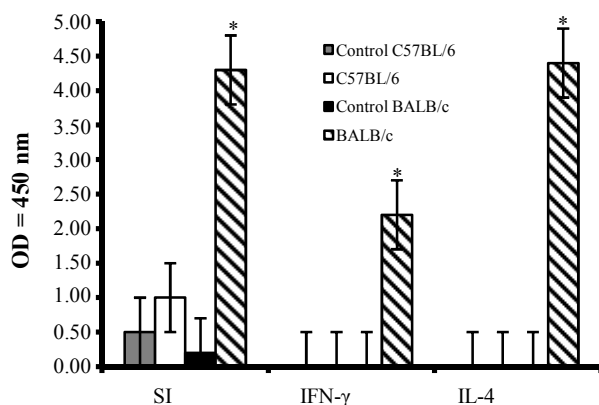


Fig. 3. Stimulation index. IFN- γ and IL-4 produced by spleen cells of mice. Data are expressed as means of triplicate experiments. Control group was comprised of serum from mice injected with PBS. Control* C57BL/6 and BALB/c mice, mice receiving PBS; C57BL/6 and BALB/c mice; mice receiving 200 μ g/day of A1-GM-CSF; SI, stimulation index. * $P < 0.05$.

concluded that A1-GM-CSF is different from DT-GM-CSF. This could be considered as an advantage for a protein with therapeutic potential. Non-specific uptake of targeted toxins by normal human tissues may cause tissue injury [12]. The development of neutralizing antibodies is detrimental to targeted toxin antitumor efficacy. In many trials, retreatments have been limited to a few cycles because of the development of neutralizing antibodies. Even when the antibodies generated are non-neutralizing, they may form immune complexes and accelerate clearance from the circulation. This antibody response also reduces clinical benefit.

This phenomenon was not observed in the case of A1-GM-CSF since the protein could not raise the high level of antibody, except in BALB/c mice that a very high concentration (200 μ g/day) of the fusion protein could raise low level of antibody. This property of A1-GM-CSF is very appealing since retreatment in many cycles can be continued without a significant risk of developing antibodies. Moreover, it was shown recently that GM-CSF itself may increase the vaccine-induced immune response at relatively low doses (40-80 μ g for 5 days), whereas an opposite effect was reported at higher doses [15]. Control of the non-specific toxicities and immune responses with various prophylactic maneuvers should further improve the therapeutic index of these molecules [12].

Due to the low, toxic effect and non-specific toxicity of A1-GM-CSF observed in this study, these preliminary data represent the A1-GM-CSF as a promising candidate for any therapeutic application.

Further studies are required to determine real clinical impact of this fusion protein.

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