Recombinant Production of IL-1Ra in Fusion to Albumin Binding Domain for Its Extended Half-Life

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Research Article

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Abstract

**Background:** Anakinra, a FDA approved biological drug for Rheumatoid Arthritis, must be injected daily due to its short Half-life, leads to the lower patient compliance. So, the aim of this study was to produce IL-1Ra in fusing to albumin binding domain to extend its half-life and evaluate its biological effects.

**Methods and Results:** The expression of IL-1Ra-ABD was performed in *E. coli* in fusing to intein1 of pTWIN1 in soluble and purified. The affinity of IL-1Ra-ABD to HSA was determined on Native-PAGE and its release percent toward time was determined. Finally, MTT assay was used to determine the antagonizing properties of recombinant IL-1Ra-ABD against IL-1β, on A375 cells. the expression induction of intein1-IL-1Ra-ABD using 0.1mM of IPTG at 15°C, and its cleavage represented a band approximately in 50 and 23 kDa respectively. Native-PAGE results showed that about 78% of IL-1Ra-ABD attached to the HSA after 2 hours of incubation, and MTT assay results showed no significant differences between the effects of our recombinant protein and native IL-1Ra.

**Conclusion:** the production of soluble IL-1Ra-ABD with similar antagonizing effects to IL-1Ra was successfully performed. IL-1Ra-ABD showed suitable interaction with HSA and release over the time. However, pharmacokinetics and further biological evaluations are required.

Introduction

IL-1Ra, known as Anakinra, was produced by Amgen Company for the first time with Kineret® as its trade name. This drug was FDA approved for the treatment of Rheumatoid Arthritis (RA), as well as Deficiency of IL-1 Receptor Antagonist (DIRA), a rare auto-inflammatory disease with involving of skin and bones [1].

This protein has 153 amino acid residues with a molecular weight of 17.3 kDa and is produced in the Escherichia coli expression system by recombinant DNA technology. The drug is recommended as SC injection of 100 mg daily with the half-life between 4 to 6 hours [2].

Rapid clearance of biomolecules from the bloodstream can limit their clinical effects and lead to the frequent administration. As mentioned, Anakinra must be SC injected daily and although, it is generally safe and well tolerated, the injection site reactions is the most concern about it and resulted in low acceptance of the patients [3]. Therefore, trying to produce derivatives with extended half-life is essential.

Pegylation is one of the most prevalent ways to extend the half-life of drugs and reduce the frequency of administration. However, due to the complexity of the pegylated drug formulations and the possibility of PEG side effects [4], alternative methods have been considered in recent years. In one study for example, it was shown that Anakinra binding to the biodegradable Hydroxy Ethyl Starch (HES) in comparison to its pegylated form resulted in increasing its thermal stability during the shelf-life [5].

Furthermore, a large number of proteins have been engineered by consensus sequence design methods with the aim of being more stable. XTEN sequence, for example, used to increase the half-life of various
recombinant drugs [6]. On the other hand, serum albumin due to the long half-life (19 days in human), the highest blood concentration compared to other plasma proteins, and the biological distribution in inflammation or tumor site, is an attractive option to increase the half-life of bio-drugs [7]. In one study, a recombinant form of IL-1Ra protein was produced in fusing to human serum albumin (HSA), and it was shown that this fusion protein is able to bind to the corresponding receptor comparable to the native protein [8]. However, it appears that the recombinant production of Anakinra fused to a large protein such as albumin may interfere with binding to its receptor, in addition to increase the gene synthesis cost and disrupt the vital processes of the host cell. So, recombinant production of drugs in fusing to the albumin binding domains (ABD) is a suitable approach to overcome these such drawbacks. There are several examples of biologic drugs which their half-lives have been increased by the addition of ABD and they are include exenatide [9], hirodin [10], and GLP1 receptor agonist [11]. In one study, several peptides with ability to bind to HSA were studied based on ABD in the G148 protein of streptococci strains, and finally a 44-amino acid peptide with eukaryotic origin with the most closely sequence to ABD was introduced [12]. In the present study, we designed Anakinra in fusion to this sequence of ABD, and recombinant production of this fusion protein with an intein tag (ssp intein) from pTWIN1 plasmid was performed followed by its purification and evaluation its antagonizing efficacy of IL-1β in comparison to the native IL-1Ra earned from our pervious study [13].

Materials And Methods

Designing and Expression of IL-1Ra-ABD fusion protein

The coding sequence of IL-1Ra was obtained from DrugBank (https://go.drugbank.com) with accession number of DBCAT002727 and fused to the amino acid sequence of ABD used in the study of Jacob et al., [3]. The three dimensional structure of IL-1Ra-ABD and salt bridge formation were predicted using Modeller 9.24 and VMD soft-wares, respectively. The pTWIN1-IL-1Ra-ABD plasmid was synthesized by BioMatik Company (Canada) after designing as fusion to intein1 of the mentioned plasmid. This construct was transformed to the E. coli BL21 (DE3) (Pasteur Institute, Tehran, Iran) as host cells, followed by selecting the recombinant colonies on LB-agar plates (HiMedia, India) containing 100 µg/mL of ampicillin (Sigma, Germany). The selected colonies were then cultivated overnight and used to inoculate fresh cultures to reach an OD600 of 0.4 to 0.6. Next, the total expression of Intein1-IL-1Ra-ABD was induced by various concentrations of IPTG (Sigma, Germany) (0.1, 0.5, 1 mM) for 4 hours at different temperatures (15, 25, and 37°C). The cells were harvested via centrifuging at 7000×g for 10 min at 4°C. Finally, the protein expression was evaluated by 12% SDS-PAGE.

Self-cleavage inducing of Ssp intein

As mentioned before, on-column cleavage and purification of IL-1Ra-ABD was mediated by the IMPACT™ (New England Biolabs, US) purification system. Briefly, the cell pellets related to the condition with the most soluble protein expression and the least auto-induce cleavage, were re-suspended in B1 buffer (Tris–HCl 20 mM, NaCl 500 mM and EDTA 1 mM, pH 8.5), and followed by sonicating for the cell
disruption. The sample was then centrifuged at 7000 × g and 10°C for 15 min and finally, the supernatant was loaded on the chitin column. The flow-through was discarded and replaced with B2 buffer (Tris–HCl 20 mM, NaCl 500 mM and EDTA 1 mM, pH 6.5). The column was incubated for 24 hours at 25°C. Eventually, various elutions were collected and analyzed by 15% SDS-PAGE. All elutions were mixed and subjected to dialysis against phosphate-buffered saline (PBS) pH 7.4 for 24 hours at 4°C. Finally, the protein concentration was determined using the Bradford method against various concentrations of human serum albumin (HSA, Octapharma, Sweden).

**Evaluation the albumin binding ability of IL-1Ra-ABD**

In order to determine the ability of the produced recombinant IL-1Ra-ABD fusion protein, to attach to the HSA, equal molar ratios of the fusion protein and HSA was mixed in PBS buffer and mixed for 2 hours on ice. The test content then transferred to an Amicon filter cut-off 30 kDa (Millipore, US) followed by centrifuging at 7000 × g for 15 min at room temperature. The supernatant as well as the flow-through were analyzed by 12% Native-PAGE.

**Evaluation the release of IL-1Ra-ABD from the HSA**

As mentioned above, the equal molar ratios of the recombinant IL-1Ra-ABD and HSA was added in the presence of PBS as the protein diluent and after immersing for the appropriate time, the sample was seeded in to the Amicon filter and subjected to the centrifuge. The flow-through was analyzed by spectrophotometry in order to determine the un-bonded protein concentration. The sample was rotated in a shaker incubator at room temperature and sampling was performed in time intervals until 50 hours. For each sampling, whole test content transferred to the Amicon filter and the flow-through was analyzed after centrifuging. Finally, the release graph of IL-1Ra-ABD was drawn based of the percent of released IL-1Ra-ABD toward the time.

**Biological Assay**

MTT assay was performed to determine the antagonizing properties of recombinant IL-1Ra-ABD, on A375 (as IL-1 receptor-positive cells) and HEK293 (as IL-1 receptor-negative cells). Briefly, 160 µl of the medium containing 3 × 10^4 cells/ml of each cell line was poured into each wells of 96 micro-plates and was incubated for 24 hours at 37 ºC. Afterward, 20 µl of the IL-1β (PeproTech, Canada) with 2 ng/ml as final concentration was added to each row of the plate except for blank. The various concentrations of the purified IL-1Ra-ABD with the final concentrations of 15, 7.5, 3.75, 1.8, and 0.94 µg/mL was added after 1 hour of incubation. After 48 hours of incubation at 37°C in 5% CO2, 20 µl of MTT (5 mg/ml) was added to each well for the other 3 hours of incubation. All well contents were replaced with 150 µl of dimethyl sulfoxide (DMSO) to dissolve formazan crystals. Finally, the plates were subjected to absorbance read at 570 nm by a microplate reader (Bio-Rad, USA). All these experiments was repeated for native IL-1Ra produced with the same procedure in the previous study instead of IL-1Ra-ABD, for both cell lines as a positive control.

**Statistical analysis**
To ensure the accuracy and reproducibility of data, the MTT test was performed for each cell line as a triple independent experiment. Cell culture media used as blank, PBS treated cells considered as negative control, and cells treated with 2 ng/ml of IL-1β, were assumed as the positive control. SPSS 25 software was used for statistical analysis. Analysis of variance (ANOVA) followed by Tukey's post hoc test were used to determine the differences between groups. P < 0.05 was considered the statistical significance of the analyses.

Results

Expression of rIL-1Ra-ABD

Expression of the Intein1-IL-1Ra-ABD fusion protein was evaluated by 12% SDS PAGE. As shown in Fig. 1, the expression induction using 1mM IPTG, revealed a band approximately in 55 kDa, for cells transformed with non-recombinant pTWIN1 plasmid which corresponds to the molecular weight of intein 1 and 2 fusion protein. For E. coli BL21 (DE3) cells containing recombinant pTWIN1-IL-1Ra-ABD, on the other hand, induction with IPTG, represents a band approximately in 50 kDa which represented the expression of IL-1Ra-ABD in fusion to intein 1. Furthermore, the best condition for protein expression and with the less auto-induced cleavage was determined as 0.1 mM of IPTG at 15°C used for about 16 hours in order earn the most soluble protein.

Purification of the rIL-1Ra-ABD

The collected fraction from the chitin column, was incubated for 24 hrs at room temperature and was analyzed on the 15% SDS-PAGE. In Fig. 2, a band of approximately 23 kDa represents the recombinant IL-1Ra-ABD. The final yield of recombinant protein production was calculated to be 4.3 mg per liter of bacterial culture medium.

Evaluation the albumin binding ability of IL-1Ra-ABD

Native-PAGE results showed that after 2 hours incubation of IL-1Ra-ABD with HSA, the binding was occurred. Actually, based on appeared bands, it was calculated that about 78 % of IL-1Ra-ABD in the test solution attached to the HAS (Fig. 3).

Evaluation the release of IL-1Ra-ABD from the HSA

Sampling from test tube containing the IL-1Ra-ABD encountered to HSA, followed by shaking at 100 RPM on an Amicon filter at 37°C showed about 80 % of release from HSA during the first 50 hours of incubation (Fig. 4a and b).

Comparison the antagonizing efficacy of IL-1Ra-ABD with IL-1Ra

One hour after the incubation of A375 cells with 2 ng/ml of IL-1β as a constant concentration, these cells were treated with increased concentrations of the recombinant IL-1Ra-ABD. The results showed that in the
similar concentration of the antagonist (15 µg/ml), there were no significant differences between the effects of our recombinant protein and alone IL-1Ra (P-value = 0.33) (Fig. 5a). For HEK293, on the other hand, IL-1β led to less survival percent of these cells and both types of antagonist (native IL-1Ra and IL-1Ra-ABD) could increase this survival; however, this effects was significantly less than A375 cells (Fig. 5b).

Discussion

In the present study, IL-1Ra in fusing to ABD was produced in E. coli expression system in order to extend the half-life of this FDA approved drug for the treatment of RA and purified using intein mediated procedure. Our results indicated that after 2 hours, about 78 % of this fusion protein can bind to HSA and released during 50 hours until it reach to a stationary phase. On the other hand, there was not significant differences between antagonistic efficacy of IL-1Ra-ABD and native IL-1Ra used as the positive control in this test when A375 and HEK293 cell lines treated by IL-1β.

Especially during the recent years, the IMPACT system usage was increased more than ever because of the intein tags ability to produce the soluble form of recombinant proteins as well as their self-cleavage ability after the protein purification. However, optimization of the conditions for soluble expression and intein cleavage is a critical role to earn the most active protein.

Lower inducer concentrations, leads to a significant reduction in the expression of recombinant protein. For example, in one study, the effect of increasing concentrations of IPTG on the expression of DT386-BR2 protein in E. coli was evaluated. The results showed that with 1 mM IPTG, the highest amount of recombinant protein production occurs but as inclusion body [13]. In the current study, to produce the soluble protein, IPTG was used in 0.1 mM concentration according to the IMPACT manual.

As another effective variable, post-induction temperature, the formation of inclusion bodies decreases in lower temperatures [14]. For example, the human G-CSF was produced in soluble form by incubating at 15°C [15].

Although, inteins usage can significantly lower the costs of producing recombinant proteins, there are several un-solved problems. The conventional intein system, raises costs due to the need for reducing agents such as dithiotreitol (DTT) [16]. Using intein1 (ssp DnaB) of the pTWIN1 plasmid, is more convenient due to induce its self-splicing activity by changing of pH, same as the previous study for purifying the recombinant human G-CSF [15]. However, another problem in this regard is the self-cleavage induction of intein1 during the recombinant production in the host cell cytosol. In order to prevent this unwanted reaction, lowering the post-induction temperature and the pH of cleavage buffer was used in this study. In general, cleavage ability can improve by increasing the temperature incubation of the chitin column. For example, in a study conducted by Tong, the best temperature was introduced as 37°C among various investigated temperatures range from 15 to 37°C [16]. also, there are several studies confirmed that lower pH to even 4.5, increasing the time of column incubation as well as the incubation temperature, leads to the efficient cleavage of intein1 [17], for example, it was established in one study for the
cleavage of a mutated form of insulin from the intein1 that pH 4.5 in comparison to the pH 8, and temperature as 25°C was more efficient in cleavage ratios. In our study, we used 25°C incubation temperature for 24 hours at pH 6.5 according to the IMPACT manual.

In one study which IL-1Ra was fused to human serum albumin, pharmacokinetic assays showed that native IL-1Ra was completely cleared from the blood circulation after 8 hours of injection to healthy mice, while for the fusion protein (IL-1Ra-ALB), their data represented about 30% of the radio-labeled fusion protein could be still observed in the circulation after 48 hours after the injection. In fact, the calculated half-life of the mentioned form of IL-1Ra was determined as about 10 hours in comparison to the native form with calculated half-life as about 0.34 hours [9]. In our study, we used ABD instead of full-length human serum to overcome the probable drawbacks of expression the protein in fusion to a large molecule.

ABD usage for extending the half-life of biological drugs was used in several studies mentioned in introduction section. For example, in attempt to increase the half-life of exenatide using ABD, the pharmacokinetics assays showed about 32-fold increasing in the half-life of exenatide-ABD when compared to native form (16 hours vs. 30 min) [10]. Our in vitro release test, confirmed that IL-1Ra fused to ABD could release from the human serum albumin until 50 hours and Native-PAGE revealed the stability of this fusion protein.

For biological assay, on the other hand, we used the potent cytotoxic and apoptotic effects of IL-1β against cells with highly expressed of IL-1R [19]. Furthermore, based on the effects of Anakinra in preventing the cytotoxic effects of IL-1β, the protocol mentioned in the methods section was used. The biological results showed more increase in the concentration of IL-1Ra-ABD, more inhibition in effects of IL-1Ra on IL-1β. In the study of Lui et al, this method was used for evaluating the inhibitory effects of a fusion protein of IL-1Ra with an extended half-life. The results showed that 32 nM of IL-1Ra led to 100% inhibition effects of 1 ng/mL IL-1β [19]. The concentrations of IL-1Ra-ABD used in the present study were higher than those investigated by Lui et al. In another study, Yu-Xin produced several mutated forms of IL-1Ra and compared their biological activities with those of native IL-1Ra. The results showed that at higher investigated concentrations (25 µg/ml), IL-1Ra can inhibit the effects of IL-1 [20].

The antagonizing efficacy of IL-1Ra-ABD was not statistically significant compared to native IL-1Ra for highly expressed IL-1 receptor cells, A375. The strength of the produced recombinant protein was about 101% of the alone form.

Lui et al., evaluated the antagonizing efficacy of the recombinant IL-1Ra produced in Pichia pastoris and said that this protein could antagonize the cytolytic activity of IL-1β (1 ng/ml) when added only after the treatment of cells by IL-1β [21]. However, in the present study, based on our previous project on evaluation the antagonizing efficacy of IL-1Ra, produced with the same protocol, a time interval of one hour was used. The results showed that IL-1Ra-ABD could successfully act in antagonizing the toxic effects of IL-1β in a time interval of 1 hour without any significant differences to native IL-1Ra.
Finally, Powers et al. used IL-1-responsive A549 cell line for evaluating the competitive antagonizing effects of a Pasylated form of IL-1Ra, produced for expanding its half-life [21]. In this study, the antagonist was added to cells and 1 hour later, IL-1α was added to the cells. The efficacy of IL-1α (in the final concentration of 10 ng/ml), in IL-6 release was evaluated and found that both Anakinra and its Pasylated form antagonized the IL-6 secretion [22]. The results of this study, also confirmed the Anakinra antagonizing effects on IL-1, only before its internalizing to the cells with highly expressed receptors on their surface.

**Conclusion**

In this project, we successfully produced and purified the recombinant IL-1Ra in fusing to the albumin binding domain as soluble form. The antagonizing effects of this protein were statistically equal to the recombinant protein produced in the same strategy. On the other hand, this fusion showed suitable interaction with human serum albumin indicated its correct three dimensional structure. Furthermore, in vitro release analysis confirmed the total release of the recombinant fusion protein from HSA. However, further in vivo pharmacokinetics analysis as well as in vitro and in vivo biological evaluations are required for this protein to act as a drug candidate for clinical trial phases.

**Declarations**

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**Competing interests**

There is no conflict of interest.

**Ethics approval**

This article does not contain any studies with human participants or animals performed by any of the authors. The Ethics Committee of Isfahan University of Medical Sciences approved this research with the code of IR.MUI.RESEARCH.REC.1399.106.
Consent to Participate (Ethics)

This article does not contain any studies with human participants performed by any of the authors.

Consent for publication

All authors are agree for publication this manuscript.

Authors' contributions

Ali Yazdani performed the experimental and wrote the first version of the manuscript, Fatemeh Shafiee designed the experimental, analyzed the data and revised the manuscript.

Availability of supporting data

The data is available and present according to the reviewer and editor comment.

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**Figures**

**Figure 1**

SDS-PAGE analysis of expression induction of IL-1Ra-ABD in various conditions regard to IPTG concentration and temperature of incubation for 4 hours of incubation. Lanes 1 and 8: protein marker, Lane 2: induced E. coli BL21(DE3) cells containing non recombinant pTWIN1 by 1 mM IPTG at 37 °C. Lane 3: induced E. coli BL21(DE3) cells containing recombinant pTWIN1-IL1-Ra-ABD, Lane 4: induced E. coli BL21(DE3) cells containing recombinant pTWIN1-IL1-Ra-ABD by 0.1 mM IPTG at 37 °C. Lane 5: induced E. coli BL21(DE3) cells containing recombinant pTWIN1-IL1-Ra-ABD by 0.5 mM IPTG at 37 °C. Lane 6: induced E. coli BL21(DE3) cells containing recombinant pTWIN1-IL1-Ra-ABD by 1 mM IPTG at 37 °C. Lane 7: induced E. coli BL21(DE3) cells containing recombinant pTWIN1-IL1-Ra-ABD by 0.1 mM IPTG at 25 °C. Lane 8: induced E. coli BL21(DE3) cells containing recombinant pTWIN1-IL1-Ra-ABD by 0.5 mM IPTG at 37 °C. Lane 9: induced E. coli BL21(DE3) cells containing recombinant pTWIN1-IL1-Ra-ABD by 1 mM IPTG at 25 °C. Lane 10: induced E. coli BL21(DE3) cells containing recombinant pTWIN1-IL1-Ra-ABD by 0.1 mM IPTG at 15 °C. Lane 11: induced E. coli BL21(DE3) cells containing recombinant pTWIN1-IL1-Ra-ABD by 0.5 mM IPTG at 15 °C. Lane 12: induced E. coli BL21(DE3) cells containing recombinant pTWIN1-IL1-Ra-ABD by 1 mM IPTG at 15 °C.
Figure 2

15% SDS-PAGE for the evaluation of the IL-1Ra-ABD cleavage. Lane 1: protein marker, Lane 2: purified recombinant IL-1Ra after the first elution of the column with B2 buffer, Lane 3: the forth elution of the column, Lane 4: the second elution of the column, Lane 5: the third elution of the column.
Figure 3

Native-PAGE for the evaluation of ABD affinity to HSA. Lane 1: protein marker, Lane 3: the mixed sample of IL-1Ra-ABD and HSA after 30 min of incubation. Lane 4: the flow-through of Amicon filter after centrifuging after 2 hours of incubation. Lane 2: the upper sample of Amicon filter after centrifuging after 2 hours of incubation.

Figure 4
a: the release percent of IL-1Ra-ABD from HSA in various times. b: Native-PAGE of the released IL-1Ra-ABD from the HAS from the flow-through of Amicon filter in various time. Lanes 1-6: the flow-through sample after 1, 3, 5, 16, 18, and 25 hours of incubation.

Figure 5

a: comparison the antagonizing effect of IL-1Ra-ABD and native IL-1Ra on A375 survival percent after the treatment with IL-1β. b. comparison the antagonizing effect of IL-1Ra-ABD and native IL-1Ra on HEK293 survival percent after the treatment with IL-1β. Error bars represent SD, n= 3. Stars show the significant differences between groups and the positive control (cells treated with IL-1β) in the same concentrations. * P< 0.05, ** P< 0.01 and *** P< 0.001.

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