Recombinant Production of a Mutant Form of Soluble IL-6 Receptor with Inhibitory Effects against Interleukin-6

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Background: Interleukin-6 (IL-6) has undeniable roles in inflammatory processes due to autoimmune diseases. In this regard, soluble receptors are considered a potential approach to mitigate its inflammatory effects and modulate its physiological effects by reducing the IL-6 binding to cell surface-specific receptors.

Objective: This study aimed to produce IL-6 receptor (IL-6R) in soluble form with enhanced affinity to IL-6 without signal transduction ability.

Materials and Methods: The 3D structure of IL-6R with the selective mutations for enhancing the IL-6 binding, with minimum ability to signal transduction (mIL-6R), was predicted using Modeller 9.19. This mutated form was docked to IL-6 and gp130 (a part of the native IL-6 receptor involved in signal transduction) by the HADDOCK2.2 web server. The expression of mIL-6R was performed in E. coli BL21 (DE3), using pTWIN-1 plasmid as its linkage to the Ssp Intein. IMPACT system manual was used to purify the protein at 25 °C overnight. Next, ELISA was performed to compare the affinity of mutated and native IL-6R to IL-6. Finally, A549 cells were used to compare the inhibition of cytotoxic effects of native and mutated IL-6R.

Results: In the silico section, results established the stability of mutant’s structure with more and less affinity to IL-6 and gp130, respectively. The expression and purification results showed bands of about 50 and 23 kDa, representing the correct size of the Intein1-mIL-6R fusion protein and cleaved mIL-6R in SDS-PAGE, respectively. Furthermore, a significant enhancement in the affinity of mutated IL-6R to IL-6 was observed compared to the native receptor. Finally, A549 cells showed more cytotoxic effects followed by treating with mutated IL-6R in comparison to cells treated with native soluble IL-6R.

Conclusion: The recombinant production of a mutated form of IL-6R with the potential ability to antagonize the IL-6 inflammatory effects confirmed with in silico studies was successfully performed for the first time to create a new drug candidate for suppressing the inflammatory effects of IL-6.

Keyword: Autoimmune diseases, A549, IL-6R, IMPACT, Recombinant production

1. Background
Rheumatoid arthritis (RA), a chronic disease with joint and systemic inflammation, results from immunological abnormalities. Although different cytokines are involved in the exacerbation of RA, the role of TNF-α and IL-6 is more than others (1). IL-6, a cytokine with 212 amino acid residues, enhances the activity of helper and cytotoxic T cells and the differentiation of Th17 involved in inflammation.

Also, this cytokine induces antibody production by B lymphocytes and causes osteoporosis and joint edema by affecting articular fibroblasts (2). One study showed an increase in the IL-6 level in patients with RA (3). Increased IL-6 levels have also been observed in mouse models prepared for RA (4, 5). Besides, the treatment of some RA patients with anti-IL-6 antibodies has led to general improvement (6, 7).

A proper approach for decreasing the IL-6 inflammatory
effects is using receptors in soluble form to modulate its physiological effects by reducing its binding to cell surface receptors. This approach has become stronger since Etanercept (Enbrel®) was developed as a TNFα modulator via binding to the cytokine and inhibiting its binding to the cell membrane receptor (8).

IL-6 exerts its effects through two different molecules: 1) IL-6Rα (gp80), known as IL-6R, which is involved in the binding of IL-6 to the membrane receptor and 2) IL-6Rβ (gp130), which is involved in the signal transduction by IL-6 (9). The soluble form of IL-6Rα is generated by proteolysis of cell surface receptors and alternative mRNA splicing (10). This antibody shows agonistic activity after binding with its ligand because of binding to gp130 (the trans-signaling pathway) (9).

2. Objectives
In the present study, we designed a mutated form of IL-6Rα with potentially enhanced affinity to IL-6, without the ability to interact with gp130 and signal transduction with several point mutations. Next, it was checked by the HADDOCK2.2 web server for comparing the interactions of the mutated and native form of IL-6R with IL-6 and gp130 too. Overall, the aim of the present study was to produce IL-6R in active soluble form with a higher affinity to attach to IL-6 with lower ability to the signal transduction. To this end, a single-step purification strategy according to the IMPACT protocol was applied, followed by evaluating its biological effects on the A549 cell line in inhibiting the proliferative effects of IL-6.

3. Materials and Methods

3.1. Bacterial Host, Gene, Plasmid, and Reagents
Various E. coli strains (TOP 10 and BL21 (DE3)) were obtained from the Pasteur Institute of Iran (Tehran, Iran). Also, pTWIN1 was from New England BioLabs (Massachusetts, USA). Ampicillin for selecting the successfully transformed colonies was purchased from Sigma (California, USA). IL-6 ELISA equipment was prepared from IBL International (Hamburg, Germany). A soluble form of IL-6Rα was purchased from MyBioSource (San Diego, California, USA). The IL-6R coding sequence was ordered to be synthesized by Zist Eghtesad Mad Company (Tehran, Iran) and received in the PUC157 plasmid. Finally, cell culture reagents or chemicals for various molecular biology tests were obtained from known related companies.

3.2. Methods

3.2.1. Theoretical Section

3.2.1.1. Selection of Mutations and Homology Modeling
The IL-6Rα amino acid sequence was obtained from NCBI (accession number: 1N26_A), and transmembrane and cytoplasmic parts were omitted. Mutations including E47L and D48G were used for increasing the affinity of IL-6Rα to its ligand, IL-6. In addition, the mutations including H167S, D168V, A169D, G172D, H175Y, and V176L were used to eliminate the attachment of a soluble receptor to gp130 (11). The three-dimensional structure of this mutated form of IL-6Rα was predicted by Modeller 9.19 using the extracellular domain of human IL-6Rα as a template (PDB code: 1N26). Among 1,000 predicted models using Modeller software, the model with the lowest DOPE (discrete optimized protein energy) score was assumed as the most suitable model for protein docking. The best model was analyzed using PROCHECK and Rampage software and Verify3D web server.

3.2.1.2. Protein Docking
The interactions of mIL-6R with IL-6 and gp130 were assessed via the HADDOCK2.2 web server and compared with these interactions in the native form of IL-6R (nIL-6R). According to the previous studies, fibronectin I and II domains interact with IL-6 (PDB code: 1ALU) and gp130 (PDB code: 1BJ8). Therefore, a truncated form containing these two domains (fibronectin I and II domains) consisting of 204 amino acid residues of IL-6R were used as passive residues, while amino acids 160-189 were used considered as active amino acids. The interactions of mIL-6R with gp130 were analyzed by considering amino acid residues in Fibronectin II of mIL-6R from 106 to 204 and assuming passive residues from 168 to 177 as active amino acids.

3.2.2. Experimental Section

3.2.2.1. Sub-cloning of the mIL-6R Coding Sequence
The coding sequence of mIL-6R was optimized for the expression in Escherichia coli. Also, the synthesized gene was sub-cloned into the pTWIN1 plasmid after the digestion with EcoRI and BamHI restriction enzymes as a fusion to Intein1 expression carboxyl end. The
cloning accuracy was surveyed by restriction digestion of the recombinant plasmid by mentioned enzymes and DNA sequencing. This recombinant plasmid was named pTWIN-mIL-6R.

3.2.2.2. Expression of Intein1-mIL-6R Fusion Protein
The BL21 (DE3) strain of E. coli was transformed with the pTWIN-mIL-6R plasmid, followed by selecting the recombinant colonies on ampicillin LB-agar plates. The selected colonies were used for inducing the protein expression. After overnight cultivation, fresh cultures were inoculated, and upon reaching an OD600 of 0.5 to 0.7, soluble expression of the Intein1-mIL-6R was induced by 0.4 mM IPTG overnight at 15°C. The cells were harvested via centrifuging at 4°C, followed by evaluating the protein expression on 12% SDS-PAGE.

3.2.2.3. Recombinant Protein Purification
Intein cleavage from mIL-6R was performed by the IMPACT™ system. Briefly, the cell pellets were re-suspended in a buffer named B1 buffer containing Tris-HCl (20 mM), NaCl (500 mM), and EDTA (1 mM; pH = 8.5). After the sonication, centrifugation was performed at 7000 × g and 4°C for 30 min. Afterward, the supernatant was loaded on the column containing chitin resin. Finally, the flow-through was collected and replaced with B2 buffer containing Tris-HCl (20 mM), NaCl (500 mM), and EDTA (1 mM; pH 6.5), inducing self-cleavage of the Intein1 and resulting in the release of the mIL-6R from the column. Afterward, the column was incubated at 25°C overnight, followed by collecting the different fractions. Finally, the collected fractions were analyzed by 15% SDS-PAGE. Bradford method was used to calculate the final concentration of the recombinant protein toward various concentrations of human serum albumin (HSA).

3.2.2.4. Evaluation of the Affinity of mIL-6R to IL-6
ELISA was used for comparing the affinity of mutated and native IL-6R to IL-6. First, the mutated or native IL-6R (MyBioSource, USA) was coated to the 96-well plate. After washing with wash buffer, the recombinant IL-6 (IBL International, Germany) was seeded to each well and incubated at 37°C for 1 h. After three times of washing, a biotin-conjugated anti-human IL-6 antibody was added to bind to human IL-6. Following incubation for another 1 h, a washing step was performed. Next, a streptavidin-HRP was added to the solution, followed by incubation for 1.5 h. After the final washing, Diaminobenzidine (DAB) solution was added to each well, and the reaction was terminated in 30 min by adding sulfuric acid. Finally, the absorbance of yellow dye was read at 405 nm using a microplate reader.

3.2.2.5. Evaluating the Inhibition Effects of mIL-6R on A549 Cells Proliferation
MTT assay was used to compare the effects of mutated and native IL-6R in inhibition of proliferative effects of IL-6 on A549 cells (12). Briefly, 2 × 10⁴ cell.mL⁻¹ of A549 suspension in RPMI 1640 was seeded in each well of a 96-well plate. After 24 h, 10 ng.mL⁻¹ of IL-6 (IBL International, Germany) was added to each well (12). Various concentrations of mIL-6R or nIL-6R (i.e., 0.1, 0.2, 0.4, 0.8, and 1.6 µg.mL⁻¹) were added to each well on the same day, and the plate was put on the CO₂ incubator for another 72 h. Finally, the plate was incubated for 3 h after adding 20 µL of MTT solution (5 mg.mL⁻¹). The formazan crystals were solubilized by adding 100 µL of DMSO and the absorbance of each well was read in 570 nm using micro-plate reader.

3.2.2.6. Statistical Analysis
Cytotoxicity test was repeated for three independent times and four wells for each protein in various concentrations. The solvent of proteins (PBS) was considered the negative control, and data were expressed as cell viability percentage ± SD (standard deviation). SPSS 23 software was used to analyze variance (ANOVA) to distinguish the differences among groups. Also, an independent T-test was used to determine the differences between two types of IL-6R for ELISA and biological assay.

4. Results

4.1. Comparing the Interactions of IL-6Rs with IL-6 and Gp130
Among 1,000 predicted structures for mIL-6R using Modeller software, one with the lowest DOPE score was selected as the best model (~205544.7832). The docking results of both forms of IL-6R with IL-6 showed more affinity of the mIL-6R to IL-6 than the native form (Table 1). Besides, it was observed that the interaction between nIL-6R and gp130 was more than mIL-6R (Table 2). These findings confirm the selected mutations for increasing the affinity of a soluble
receptor with its ligand and diminishing its affinity to form a dimer with gp130 and signal transduction.

4.2. Cloning and Expression of Recombinant mIL-6R

Successful mIL-6R in the fusion with the Intein1 sub-cloning was established by restriction enzyme digestion and DNA sequencing, as mentioned in the method section. The expression of the Intein1-mIL-6R fusion protein was evaluated by 12% SDS-PAGE, where a band of about 50 kDa revealed the Intein1 fused to mIL-6R expression. However, in the case of the expression induction on bacteria-harboring non-recombinant pTWIN1 vector, a band with 55-kDa size contributing to the size of the fused Intein 1 and Intein 2 was observed (Fig. 1).

Furthermore, the best culture conditions for producing the protein in soluble form were determined as 0.4 mM IPTG, temperature = 15 °C, and incubation time overnight (Fig. 1)

4.3. On-column Purification of the Recombinant mIL-6R

As shown in Fig. 2, 15% SDS-PAGE of the fraction collected 24 h following incubation of the recombinant protein bound to the chitin column with buffer B2 at room temperature revealed a band of about 23 kDa, pertaining to the mIL-6R molecular weight. The total recombinant protein purified in soluble form was calculated as about 2.3 mg. L⁻¹ of medium.

4.4. Evaluation of the mIL-6R Affinity to IL-6

The same molar ratios of mIL-6R and nIL-6R (100

| Parameters                        | Mutated IL-6R-IL6 complex | Native IL-6R-IL6 complex |
|-----------------------------------|---------------------------|--------------------------|
| HADDOCK score                     | -67.5 ± 13                | -62 ± 18                 |
| Cluster size                      | 5                         | 4                        |
| RMSD from the overall lowest-energy structure | 0.6 ± 0.3                | 13.7± 0.2                |
| Electrostatic energy              | -430.5 ± 27.8             | -349.3 ± 82.3            |
| Van der waals energy              | -90.2± 4.4                | -82.6 ± 3.8              |
| Desolvation energy                | 44.1 ± 3.2                | 23.8 ± 10.2              |
| Restraints violation energy       | 646.8 ± 103.5             | 667.3 ± 96.15            |
| Buried surface energy             | 2674.1 ± 88.3             | 2698.9 ± 167.7           |
| Z-score                           | -1                        | -1.4                     |

| Parameters                        | Mutated IL-6R-gp130 complex | Native IL-6R-gp130 complex |
|-----------------------------------|-----------------------------|-----------------------------|
| HADDOCK score                     | -86.1 ± 36.5                | -91.8 ± 36.0                |
| Cluster size                      | 6                           | 4                           |
| RMSD from the overall lowest-energy structure | 0.7 ± 0.4                | 1.0 ± 0.7                   |
| Electrostatic energy              | -572.5 ± 99.0               | -346.6 ± 95.1               |
| Van der waals energy              | -92.6 ± 17.6                | -104.4 ± 9.6                |
| Desolvation energy                | 33.3 ± 5.8                  | -8.2 ± 7.0                  |
| Restraints violation energy       | 847.3 ± 174.55              | 901.9 ± 184.14              |
| Buried surface energy             | 2977.3 ± 257.8              | 2832.8 ± 301.4              |
| Z-score                           | -1.6                        | -2.4                        |
ng.mL⁻¹) showed various absorbances in a wavelength of 405 nm, confirming the difference in the binding ability of IL-6. In this study, the independent T-test showed a statistically significant difference between the absorbance of two these proteins in the same concentration (0.938 and 0.653 in average for mIL-6R and nIL-6R, respectively), confirming the higher ability of mIL-6R to bind to IL-6 (p = 0.003).

Figure 1. 12 % SDS-PAGE of expressed mutated form of IL-6R in various conditions: Lane 1. E. coli BL21 (DE3) transformed with non-recombinant pTWIN1 plasmid. Lane 2. PageRuler™ unstained protein marker 26610. Lane 3. E. coli BL21 (DE3) transformed with pTWIN1 – IL-6R with 1 mM IPTG at 37 °C. Lane 4. E. coli BL21 (DE3) transformed with pTWIN1 – IL-6R with 0.4 mM IPTG at 15 °C (total protein). Lane 4. E. coli BL21 (DE3) transformed with pTWIN1 – IL-6R with 0.4 mM IPTG at 15 °C (soluble protein).

Figure 2. 15 % SDS-PAGE of expressed mutated form of IL-6R. lane 1. PageRuler™ unstained protein marker 26610. Lanes 2-4. Elution 3 to 1 of purified IL-6R.

Figure 3. comparison the effect of mIL-6Ra and nIL-6R on A549 survival percent after the treatment with IL-6. Error bars represent SD, n= 3. Stars show the significant differences between groups in the same concentrations. * P< 0.05, ** P< 0.01 and *** P< 0.001.
4.5. Evaluation of the Inhibition Effects of mIL-6R on A549 Cells

The statistical analysis results established that increasing the concentrations of nIL-6R resulted in increased cell survival in a concentration-dependent manner. In fact, using nIL-6R in 0.2 µg.mL⁻¹ and higher showed significant effects in cell survival compared to cells treated with the IL-6 (10 ng.mL⁻¹) alone. For mIL-6R treated cells, on the other hand, Tukey’s post hoc test showed that all concentrations of mIL-6R decreased the cell content compared to the IL-6 treated cells (p < 0.05) (Fig. 3). Furthermore, T-test results showed significant differences between the cells treated with the same concentration of native or mutated IL-6R (p < 0.05).

5. Discussion

Most drugs currently used for IL-6 inhibiting are from the monoclonal antibody family, which its production and purification require large equipment and costs (13). Drug production and purification at a lower cost can facilitate access to medicines for all patients and reduce drug discontinuation due to the high cost. This project aimed to introduce a new candidate drug produced in a high-efficiency and straightforward procedure with a greater affinity to bind to IL-6 compared to the native soluble receptor. This drug is without the ability to activate intracellular signaling pathways followed by IL-6 binding.

The first study to investigate amino acids involved in binding IL-6R to its ligand and interacting with gp130 was performed by Yawata et al. They showed that L247, F248, and R250 are involved in strongly binding of IL-6R to IL-6 (11).

The critical role of mentioned arginine (R138 in mIL-6R) in its interaction with IL-6 was affirmed in another study (11). Therefore, these amino acids remained preserved in our sequence. The presence of amino acids L254, Q255, H256, and C258 also interacted with different amino acids in IL-6 and their presence is essential for this interaction (14). In this respect, Yawata et al. revealed that changes in the amino acids Q255 and H256 reduced the receptor’s affinity for IL-6 (11). Therefore, these amino acids also remained intact in the protein sequence designed for this study.

Based on all these findings, all essential amino acids remained at the receptor involved in ligand interaction and L47 and G48, increasing the flexibility of the receptor tending to ligands. Eventually, the receptor interaction with its ligand was used in the final sequence. In Yawata’s study, the amino acids involved in binding IL-6R to gp130 also were identified. By changing these amino acids, the affinity to gp130 binding can be decreased. We observed that the affinity of nIL-6R for binding to gp130 and signal transduction is greater than mIL-6R. Our in-silico evaluation demonstrated that the interaction between IL-6R and gp130 is mostly done through electrostatic attractions. Therefore, a change in the overall charge of the region involved in attachment to gp130 helps reduce its affinity to gp130. According to our results, the pKa of the region involved in binding to gp130 is equal to 7 and 4 for nIL-6R and mIL-6R, respectively. This decrease in tendency can be attributed to the addition of more acidic amino acids and changes in the total charge of this region.

The pTWIN1 plasmid can be used to express recombinant proteins in soluble form. For example, in one study, an anticoagulant peptide related to Ancylostoma caninum was expressed as a soluble protein in fusion to Intein 2 (Mxe mycobacterium xenopi), i.e., pTWIN1 (15). In another study, the expression of the OG2 antimicrobial protein was performed using this vector as its attachment to Intein 2 (16); a high level of soluble protein was obtained in IPTG concentration of 0.05 mM and temperature of 37 °C for 3 h (16). However, the linkage of proteins to Intein 2 induced its self-cleavage ability by reducing agents such as Dithiothreitol (DTT). As a result, it enhances the price and complication of the purification and the 3D conformational changes of proteins with a disulfide bond. Accordingly, attaching a protein-coding sequence to Intein 1 of pTWIN1 eliminates agent addition and simplifies the purification process.

In Yang et al.’s study, attachment of IL-10 fused to RGD motif was performed to the Intein 1 of pTWIN1 and expressed as inclusion body at 37 °C for 5 h and IPTG in final concentration as 1 mM (17). Declining incubation temperature after induction and decreasing inducer concentration can slow down inclusion bodies formation (18). According to these studies and based on the IMPACT protocol, we used 15 °C as incubation temperature for 16 h and obtained the highest amounts of soluble protein.

Soluble IL-6R in cells without expression of IL-6Ra but with gp130 on their surface led to response to IL-6 in Jurkat cells (IL-6Ra) in a gp130 concentration-dependent manner (10). In other words, the soluble IL-6Ra can attach to IL-6 and then attach to the gp130.
on the cell surface, resulting in signal transduction. In the present study, the affinity of IL-6R to gp130 was diminished using point mutations established in Yawata’s study (11). A549 cells have been used for evaluating the signaling inhibition effects of mutated IL-6R. The results showed that IL-6 enhances the survival of malignant cells transfected with the coding sequence of its gene (19). In addition, IL-6 leads to DNA repair and apoptosis prevention in A549 cells (20). In one study, a lentiviral vector containing IL-6 siRNA was transfected to A549 cells after encountering ionizing radiation. This study showed that the survival of these cells was lower than cells expressing IL-6. Actually, IL-6 played a role in protecting A549 cells from radiation-induced DNA damage and apoptosis (20).

Another study investigated the effects of 50 ng.mL\(^{-1}\) of exogenous IL-6 for 24 h followed by adding 100 µg.mL\(^{-1}\) of tocilizumab in combination with 40 µg.mL\(^{-1}\) of 5-FU to target colon cancer cells (21). Based on the obtained results, tocilizumab could successfully neutralize the proliferation properties of IL-6 in these cancer cells when used in the mentioned concentration. Finally, Lee et al. showed the proliferative effect of IL-6 on A549 cells. They used IL-6 with 10 ng.mL\(^{-1}\) for cells as a concentration with the highest effect of cell count after 5 days (12). Therefore, this constant concentration of IL-6 was used regarding its significant proliferation effects on A549 cells. The addition of mutated or native IL-6R was performed in the same concentration, and the finding of our study was consistent with previous studies. As shown in the results section, in 0.2 µg.mL\(^{-1}\) of the IL-6R, the constructed mutated form could significantly neutralize the proliferative effects of IL-6. Despite the antagonizing effect of tocilizumab reported by Wing et al. in the concentration of 0.6 µM (21), our designed protein could decline the effects of IL-6 to half in the lower concentrations at about 0.07. Finally, a comparison was made between the reactions of cells treated with nIL-6R and mIL-6R after encountering with the same concentration of IL-6. Based on the outcomes, although the native form of the receptor could not successfully augment the proliferative effects of the IL-6, the mutated form could decrease the mentioned effects even to half-fold when treated with a concentration of about 1.6 µg.mL\(^{-1}\).

6. Conclusion
The present study could successfully produce a recombinant form of the soluble IL-6 receptor with potential higher affinity to free IL-6 and in the soluble and probably active form. Also, it can be purified using a single-step procedure with the final yield as 2.3 mg.L\(^{-1}\). Furthermore, the affinity of IL-6 to the mutated soluble receptor was investigated using ELISA assay, which confirmed its preventive properties on the proliferation effects of IL-6 on A549 cells. However, since several studies showed that elevated levels of the IL-6 have tumor-promoting activity and tumor development, it is concluded that mL-6R can act as an anti-cancer drug candidate. Hence, the cytotoxicity evaluation of this recombinant protein against various cell lines should be considered in future studies.

Consent for publication
All authors are agree for publication this manuscript.

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

Competing interests
There is no conflict of interest.

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Authors’ contributions
SF performed the experimental and wrote the first version of the manuscript, FSh designed the experimental, analyzed the data and revised the manuscript.

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