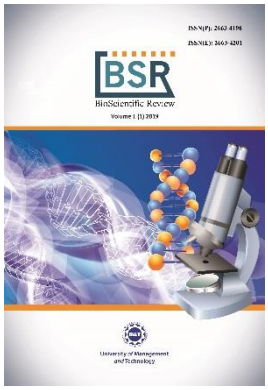

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Research Article

Studying the pharmacokinetics of interleukin-1RA mutants

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Abstract

The two antagonists for interleukin-1 (IL-1) system are IL-1α, and IL-1β. IL-1 receptor antagonist (IL-1RA) is another naturally occurring non-competitive antagonist. All three antagonists are bound to two receptors that are IL-R1 and IL-1R2. IL-1 is a well-studied pro-inflammatory cytokine that is involved in chronic inflammatory diseases such as rheumatoid arthritis, Parkinson's disease, Alzheimer's, stroke, and epilepsy. The existing literature suggests that interleukin-1 (IL-1) biological responses are impeded by interleukin-1 receptor antagonist (IL-1RA) belonging to interleukin-1 (IL-1) family and a natural IL-1 inhibitor acting as 'receptor antagonist'. Various studies based on different clinical and experimental models suggest that IL-antagonism has been reported beneficial in neuropathalogical conditions, whereas, pro-inflammatory actions of IL-1 receptor antagonist (IL-1RA) seem detrimental. This study was performed to ameliorate the stability of IL-1RA mutants generated by mutagenesis at specific sites like $R^{6}K^{7}$-AA, $R^{93}K^{94}$-AA and $K^{97}R^{98}$-AA. Using pTIG-Trx expression system, mutants were expressed in *E. coli* BL21 (DE3) accompanied with the induction of (isopropyl β-D-1-thiogalactopyranoside) IPTG. Later on, the purification of recombinant proteins was brought about using SephadexG75 gel filtration chromatography and Ni^{+2} chelate chromatography. The bioactivity assay result exhibited IL-IRA activity as high as mutants' activity. The characterization of pharmacokinetic profile of IL-1RA apace with its mutants presents the half-life of third mutants 2.11 times longer than that of wt IL-1RA. This experimental study will lead to novel approaches, experiences and baseline data, thus providing basis for further research to outdo and elevate the metabolic stability of IL-1RA.

Keywords: IL-IRA, Ptig-Trx, IPTG, Mutants, IL-1 receptor

1. Introduction

Interleukin 1 (IL-1) belongs to the family of cytokines and consists of two potent inhibitors of immune and inflammatory responses IL-1α and IL-1β. Naturally occurring IL-1 receptor antagonist (IL-1RA) that acts as the inhibitor of IL-1 responses is considered as the third member of this family. IL-1α and IL-1β binds with IL-1RA receptor (80-kDa) found on neutrophils and B cells. Likewise, IL-1α and IL-1β also binds with another 80-kDa cell surface receptor exclusively found on neutorphils and B cells. Loss of function mutations in IL1RN or deficiency of the IL-1 receptor antagonist (DIRA) cause a rare autoinflammatory disease characterized by unopposed activation of the IL-1 pathway (1, 2).

Interleukin-1 receptor antagonist (IL-1RA) belonging to superfamily interleukin-1 (IL-1) (3) possesses a high affinity for IL-1 receptor and specifically bind to it without provoking signal transduction, thus serves as an endogenous inhibitor of IL-1. This interaction of interleukin-1 receptor antagonist (IL-1RA) with IL-1 receptor leads to the obstruction of activities of IL-1 (4). Several researchers have demonstrated that IL-1, an eminent cytokine which enfeebles cartilage by triggering the release of prostaglandin E2 (PGE2) and metalloproteinases, increases bone

resorption by triggering osteoclast differentiation and activation and hence contributes towards rheumatoid arthritis. Moreover, the destructive changes observed when animals' knees were injected with intra-articular IL-1 were actually the clinical manifestation of arthritis (5). The recombinant IL-1RA (rhIL-1RA, biopharmaceutical drug Kineret) was investigated in clinical trials dealing with patients infected with rheumatoid arthritis. Nevertheless, the limitation or flaw associated with this therapy is the copious amount of dosage which is 100-150 mg/day, because this quantity is abundant enough to affect the psychological, financial and physical conditions of patients.

The therapeutic potential of IL-1RA has been analyzed in humans, animal models, studies and in vitro. The two most credible drug governing bodies 'US Food and Drug Administration' and 'European Agency for the Evaluation of Medicine Procedure' approved Kineret during the years 2011 and 2012. Other than one N-terminal methionine, Kineret was similar to natural nonglycosylated form of IL-1RA. A clinical trial-based study revealed that Kineret is not a placebo, instead it has proved itself an impeccable clinical therapeutic agent to treat the disease. But to the researcher's dismay, a plethora of IL-1RA was needed (approx. 10,000 to 100,000 times more) because minimal amount of IL-1 is sufficient to provoke a thorough biological response (6). Likewise, to acquire effective and successful clinical results, frequent administration and high dose is also required. This leads to homeostasis disturbance accompanied with alterations in physical, mental, behavioral and financial conditions.

Proteases are among several determinants which negatively affect the innate metabolic stability and can degrade proteins and peptides rapidly through catalytic mechanism, thus play a pivotal role in protein metabolism. Residue replacement at metabolically vulnerable sites can improve metabolic stability, therefore, this study aims at improving the metabolic stability via site-specific mutagenesis. Two adjacent basic residues involved in many active reaction sites of proteases were replaced with alanine residue. Enhanced stability of protein was observed in mutant ($K^{97}R^{98}$-AA) followed by a prolonged half-life (5.98 ±0.38) that is 2.11 times more than the half-life of IL-1RA.

This study is designed to accomplish two aims based on genetic engineering which are given below.

1. The extension of IL-1RA half-life.
2. The improvement of IL-1RA metabolic stability.

2. Materials and methods

2.1. Reagents, Bacterial Strains, and Cell Lines

Restriction enzymes including *Eco R1*, *Xho 1*, DNA Marker DL2000, *Taq Polymerase*, goat antihuman IL-1RA polyclonal antibodies, horseradish peroxidase-conjugated rabbit antigoat IgG and bioantigoat IgG, streptavidin-horseradish peroxidase conjugate, low molecular weight protein marker, almodulin A23187, BenchMark™ prestained protein ladder, recombinant human IL-2.

Bacterial strain, plasmid and cell lines including E.coli *BL21*, plasmid pTIG-Trx, mouse thymic lymphoma cell, EL-4, mouse lymphocyte cell and CTLL-2 cell lines were used.

2.2. Site-Specific Mutagenesis

Preserved IL-1RA gene isolated from pBV220 vector was cloned using PCR having two primers. The overlapping extension of PCR-mediated mutagenesis gave rise to three mutants IL-1RA, IL-1RA-1, IL-1RA-2 and IL-1RA-3. Mutation site IL-1RA-1 was near to 5' end and P_{1-2} acted as the overlapping internal primer. Mutations belonging to both IL-1RA-2 and IL-1RA-3 mutants were designed on the basis of two overlapping internal primers: P_{2-1} and $P_{2-2,}$ P_{3-1} and P_{3-2}. One-step PCR with two primers P_{1-2} and P_{28} was performed to generate IL-1RA-1. Mutant fragments IL-1RA-2 and IL-1RA-3 were amplified by two-step PCR using the same two external primers P_{27} ad P_{28} for both mutants. The two DNA fragments generated during the first stage were mingled to design the template for the second stage. PCR processing involved denaturation (at 94ºC for 5 min preceded by 25 cycles of denaturation for 30 seconds), annealing (at 50 ºC for 30 seconds), extension at 72 ºC for I min) and ended up with final extension (at 72 ºC for more 7 minutes).

2.3. Plasmid Construction

PCR product digestion was manipulated by two restriction enzymes *Eco* R 1 and *Xho* 1. The digested PCR product was cloned in an expression vector pTIG-Trx cleaved by the same restriction enzymes. The expression vector was then transformed in *E. coli* BL21 (DE3). The genome of selected transformants were sequenced afterwards for confirmation (7).

2.4. SDS-PAGE Analysis Of IL-1RA and Its Mutants in BL21 (DE3)

The expression was analyzed when positive transformants carrying the expression vector with IL-1RA or its mutant gene were cultured in an LB medium augmented with 100mg/L Amp at 37ºC, and the cultures were induced with 0.3mmol/L IPTG at 20 ºC. About six hours after induction the cells were harvested, followed by SDS-PAGE performed to analyze whole cell-lysates (8).

2.5. Western Blot Analysis

Proteins separated as a result of SDS-PAGE were transferred to PVDF membrane, 5% BSA in western wash buffer that impeded the filters followed by subsequent reactions with horseradish peroxidase-conjugated rabbit antigoat IgG and goat antihuman IL-1RA polyclonal antibody. DAB generated the signals (9).

2.6. Purification of recombinant IL-1RA and its three mutants

2.6.1. Ni^{+2} Chelate Chromatography

Proteins were produced when positive transformants carrying the expression vector with IL-1RA or its mutant gene were cultivated by high cell-density culture. The cultivated cells were centrifuged to make them harvested. Cells were lysed by ultrasonication and then purification was done by treating supernatant with Ni^{+2} chelate chromatography column balanced by binding buffer (2.3g Na_2HPO_4 + 29.22g NaCl + 0.456g NaH_2PO_4 + 1000mL H_2O). Proteins were eluted by binding buffer containing 0.5 mol/L of imidazole and fractions of proteins were intermixed (10).

2.6.2. Sephadex G75 gel filtration

Purified proteins obtained as a result of Ni^{+2} chelate chromatography were loaded on Sephadex G75 gel filtration using an AKTA Purifier system. The column was balanced or equilibrated and then washed with PBS buffer (11). Subsequently, the purified protein was resolved and analyzed on SDS-PAGE.

2.7. Bioassay Of IL-1RA and Its Mutants

Interleukin-2 (IL-2) was generated by EL-4 cells' stimulation with IL-1 and IL-1RA. Hence, the performance, efficiency and activity of IL-1RA and its mutants was measured indirectly by the proliferation of CTLL-2 cell belonging to IL-2-dependent murine T-cell line. Serial dilutions of purified IL-1 and its mutants were prepared to the final concentrations of 0.0025, 0.005, 0.025, 0.25, 2.5 and 25 μg/mL in 96 well microplates. Each well actually incorporated 50μL IL-1ra or its mutants, 100 μ L IL-1 (10 u/mL), 100 μ L EL-4 cell (2×10^6 cell/mL), 5×10^{-7} mol/L calmodulin A23187 and final volume was 250μL. This step was followed by overnight incubation at 37ºC. Then, 100 μL of supernatant from each well was transferred to 100μL CTLL-2 cell culture (1×10^5 cell/mL). The CTLL-2 cells were incubated at 37ºC for 20 hours followed by staining with 10 μL MTT (5 μ g/mL) for 4 hours at the same temperature. About 180 μL of supernatant was discarded and the rest was introduced to 100 μL isopropyl alcohol with 0.04 mol/L hydrochloric acid and the signal was finally measured at 570nm.

2.8. Preliminary Pharmacokinetics of IL-1RA and Its Mutants

This study employed subcutaneous injection into female rabbit for the characterization of pharmacokinetic profile of IL-1RA and its mutants. Predose and postdose blood samples at 0.5, 1, 2, 3, 4, 5, 6, 8, 12, 24, 36 and 48 respectively were collected for PK test. About 500μL of whole blood was taken in 1% Heparin-containing EP tubes and centrifuged 10,000 revolution per minute for 1 minute and 4ºC with plasma collection at regular intervals. Specimens were preserved at -20ºC.

Enzyme-linked immunosorbent assay having lower limit 100 ng/mL was performed to quantify plasma concentration of IL-1RA and its mutant. All wells of the 96 well microplate were coated with plasma samples obtained from PK experiments and IL-1RA standard specimens in rabbit plasma. The addition of 2% BSA blocked the plates, then the goat anti-human IL-1RA polyclonal antibody, bioantigoat IgG, and streptavidin-horseradish peroxidase conjugate were added in a successive manner. The signal was detected and measured at 570nm.

PK analysis of IL-1RA and its mutants was performed using software 3p97.

3. Results

3.1. Site-specific mutagenesis

One step PCR was performed to amplify IL-1RA and IL-1RA-1 likewise, other mutants were amplified by two step PCR. The resulting sequences of 479 residues were observed on gel electrophoresis.

3.2. SDS-PAGE analysis of IL-1RA and its mutants in BL21 (DE3)

The expression of IL-1RA and its mutants was observed as per the aforementioned protocol. The results of SDS-PAGE exhibited a high level of proteins expressed in BL21 (DE3). After cell lysis by ultrasonication, SDS-PAGE analysis of supernatant and precipitate revealed that IL-1RA and its mutants were present in supernatant.

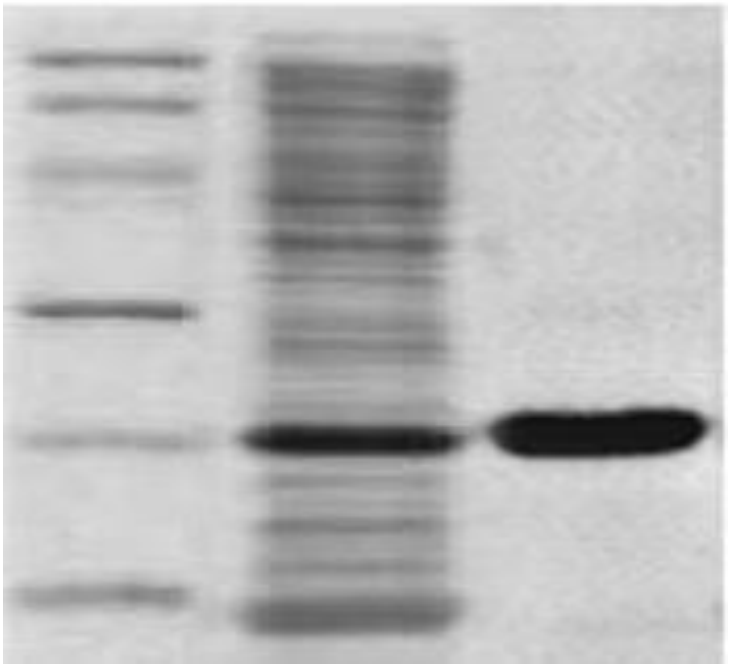

Figure 1. SDS-PAGE analysis of purified IL-1ra.

Low-molecular weight protein marker and supernatant protein of BL21 (DE3)/pTIG-Trx-IL-1ra were loaded in well 1 and 2. Well 3 shows purified IL-1ra.

3.3. Western blot analysis

Western blot analysis showed that IL-1RA and its mutants reacted with IL-1RA antibody.

3.4. Purification of Recombinant IL-1RA and Its Three Mutants

The result of SDS-PAGE is shown in Figure 1. First well is loaded with low molecular weight protein marker, second well shows BL21 (DE3)/pTIG-Trx-IL-1RA, whereas the band in the third well indicates purified IL-1RA.

3.5. Bioassay of IL-1RA and Its Mutants

Bioassay results presented that the survival rate of IL-1RA and its mutants lead to significant decline in the survival rate of CTLL-2 cells in a dose-dependent fashion. According to the statistical analysis, no considerable difference between IL-1RA and its mutants was observed (P= 0.2248).

3.6. Preliminary PK Analysis Of IL-1RA and Its Mutants

Enzyme-linked immunosorbent assay having lower limit 100ng/mL assisted us to quantify IL-1RA and the plasma concentration of its mutants. The values which decreased the lowest limit of quantification were considered zero. The T_{max} accessed its peak at about 3h of postdose in response to s.c. injection of 1 mg/kg IL-1RA or its mutants but the concentration of plasma dropped off thereafter. When the PK of IL-1RA and IL-1RA-1 were compared, no considerable difference was observed among T1/2 (K_c) of IL-1RA, IL-1RA-1, and IL-1RA-2. However, the data suggested that T1/2 (K_c) of IL-1RA-3 was (5.98 ± 0.38) h, which was 2.11 times longer than IL-1RA. Hence, the results suggested improvement in the metabolic stability of IL-1RA-3.

4. Discussion

Deficiency of the IL-1 receptor antagonist (DIRA) causes a rare inflammatory disease that occurs because of the loss of function mutations resulting in an unopposed activation of the IL-1 pathway. Ender Altiok reported a novel nonsense mutation in IL1RN gene in a prematurely born baby accompanied with intrauterine onset, death and multiorgan involvement. The study further revealed the association of novel Q119X mutation with the impaired ability of IL-1RA to antagonize signaling through the IL-1R (12).

While research work was being performed, mutant IL-1RA was developed firstly through site-specific mutagenesis that targeted $R^{6}K^{7}$-AA, $R^{93}K^{94}$-AA and $K^{97}R^{98}$-AA sites.

The findings of our study are almost similar to findings of Wang YX et al., which also showed the activity of mutants as high as IL-1RA. The half-life of the third mutant was found 2.11 times as compared to wt IL-1RA (13).

The activity of IL-1 has been reported to modulate by two different modes of regulation. IL-1RA blocks the binding of IL-1 to Il-1RA signaling. Another mechanism of regulation defined in the literature is the decoy receptor or IL-1R2 that has the ability to bind to both IL-1 accessory protein and IL-1 resulting in blocked interaction with IL-1R1 that is an active receptor. Moreover, systemic IL-1 activity is modulated by release and cleavage of soluble IL-1R2 into the circulation (14).

Dahlen et al. developed IL-1RA mutants with enhanced antagonistic activity and observed the range of mutations that increased the antagonistic function. E90Y, E52R, and D47N were identified as the most important for the effect, whereas, M136N, Q129L, H54R, and P38Y were associated with increased antagonistic function. It was further reported that mutations associated with an increased antagonistic activity enhanced the binding potential for IL-1R or had a stabilizing effect on the IL-1RA mutants. Their study demonstrated that the antagonistic activity of IL-1RA mutants was increased up to 9 times (15).

5. Conclusion

The results of PK analysis exhibited that the half-life of one mutant $K^{97}R^{98}$-AA was prolonged and showed a significant increase of 2.11 times than that of IL-1RA. This study also revealed that the substitution of unstable residue at metabolically vulnerable sites by genetic engineering led to a momentous improvement in metabolic stability. This study will be processed further in future to come up with scientific breakthroughs like novel drug delivery systems (e.g., liposomal preparations and controlled-release formulations) and chemical alteration (e.g., pegylation).

Competing interest

None

Funding

None

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