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Novel multimeric IL-1 receptor antagonist for the treatment of rheumatoid arthritis

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ABSTRACT

Protein therapeutics targeting inflammatory mediators have shown great promise for the treatment of autoimmunities such as rheumatoid arthritis (RA). However, a significant challenge in this area has been their low *in vivo* stability and consequently their severely compromised therapeutic efficacy. One such therapeutic molecule IL-1 receptor antagonist (IL-1ra), used in the treatment of rheumatoid arthritis, has displayed only modest efficacy in human clinical trials owing to its short biological half-life. Herein, we report a novel approach to conglomerate individual protein entities into a drug depot by incorporation of an amyloidogenic motif Lys-Phe-Phe-Glu (KFFE) thereby dramatically improving their systemic persistence and in turn their therapeutic efficacy in a mice model of autoimmune arthritis.

1. Introduction

Immunomodulatory proteins or peptides are preferred for the treatment of various autoimmune diseases such as rheumatoid arthritis (RA), multiple sclerosis (MS), systemic lupus erythematosus (SLE), etc. However, despite being specific and non-toxic, it is their moderate efficacy owing to their labile nature in proteolytic environments which remains to be addressed.

IL-1 receptor antagonist (IL-1ra) is a naturally occurring endogenous protein molecule that neutralizes the effects of Interleukin-1 (IL-1), a pro-inflammatory cytokine widely implicated in the pathogenesis of several inflammatory disorders [1–3]. Recombinant IL-1ra or anakinra (generic name) is currently used for the treatment of RA [4,5] which is a crippling autoimmune disease wherein the immune system principally attacks the joints [6]. The beneficial effects of IL-1ra in human subjects and animal models include downregulation of matrix metalloproteinases, promotion of cartilage repair by induction of collagen and glycosaminoglycan synthesis and consequent retardation of radiographic disease progression [5,7]. Regardless of its overwhelming benefits in experimental animal models, an excellent safety profile and effectiveness in disorders such as systemic juvenile idiopathic arthritis [8,9], auto-inflammatory Muckle-Wells syndrome [10] and adult-onset Still's disease [11,12] the performance of IL-1ra in clinical trials conducted in RA patients has been rather modest and disappointing when compared to anti-TNF biologicals [13–15].

The unsatisfactory clinical response to IL-1ra is attributable to its short biological half life (~4–6 h) limiting the sustenance of efficacious drug levels to short durations i.e. only 3–7 h in circulation [16]. Further, a daily dosing regimen predisposes the treated individuals to the development of injection-site reactions thus making patient compliance difficult [4]. So, better delivery systems for IL-1ra are certainly warranted.

The relatively short biological half life of IL-1ra appears to be a direct consequence of proteolytic degradation of individual protein molecules which disperse rapidly into circulation following administration; an efficacy limiting event common to most of the peptide and protein therapeutics. To address the issue of limited biological half life a possible strategy could be to hold or bundle individual protein molecules into a higher order assembly also acting as a reservoir which releases protein monomers gradually instead of a burst(s).

The potential of protein aggregates or amyloids to serve as drug depots has been evaluated in case of gonadotropin-releasing hormone [17] and insulin [18] wherein their inherent property to aggregate and generate complex molecular assemblies at physiological conditions was exploited. However, not all proteins aggregate at physiological conditions as was seen in the case of IL-1ra.

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Since IL-1ra does not aggregate under physiological conditions and further aggregates obtained under harsh conditions were therapeutically inactive therefore in the present study IL-1ra was engineered with the aim to improve its clinical potential. For this, an amyloidogenic motif (Lys-Phe-Phe-Glu; KFFE) was incorporated into IL-1ra. The variants generated namely, IL-1ra1, IL-1ra2 and IL-1ra3 were fusion proteins of IL-1ra and KFFE. The variants displayed superior multimerisation kinetics under near physiological conditions. Of the three variants, IL-1ra1 displayed functionality at par with native IL-1ra. A single injection of multimeric IL-1ra1 was significantly effective in reducing the clinical signs and symptoms of inflammation in a mouse model of RA i.e. collagen-induced arthritis (CIA). It also reduced the circulating levels of various pro-inflammatory markers along with a dramatic alteration in the radiographic progression of the disease. Thus multimeric IL-1ra reported herein is capable of maintaining efficacious blood levels of IL-1ra for longer durations with simultaneous elimination of the need for multiple-injections.

2. Materials and methods

2.1. Cloning expression and purification of IL-1ra and variants

THP-1 human monocytic cells (American Type Culture Collection (ATCC)) were stimulated with 1 ng/ml lipopolysaccharide *Escherichia coli* O111:B4 (LPS, Sigma, USA) and 100 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma). Total RNA was prepared using GenElute mammalian total RNA miniprep kit (Sigma) as per manufacturer's protocol. Total RNA thus obtained was reverse transcribed into cDNA with random hexamers employing Protoscript first strand cDNA synthesis kit (New England Biolabs) following manufacturer's instructions. Subsequently, cDNA was amplified using gene specific primers namely: Primer 1 – A A G C T T T G C G A C C C T C T G G G A G A A A A T C C, Primer 2 – A A T T C T T A C T C G T C C T C C T G G A A G T A G A A T T T G G, Primer 3 – A A T T C T T A T T T A A A A A A T T C C T C G T C C T C C T G G A A G T A G A A T T T G G, Primer 4 – A A G C T T T G A A A T T T T T T G A A C G A C C C C T C G G G A G A A A A T C C. Primer pairs 1 (forward), 2 (reverse); 1 (forward), 3 (reverse); 4 (forward), 3 (reverse); 4 (forward), 2 (reverse) were employed to generate IL-1ra; IL-1ra1; IL-1ra2 and IL-1ra3 amplicons respectively. Amplified products were cloned, expressed and purified using Profinity eXact tag expression system (Biorad) as per manufacturer's protocol. The sequences of IL-lra (accession no. NM 173842) and its variants (IL-lra1, IL-lra2, IL-lra3) cloned were verified by DNA sequencing. Identity of the proteins purified was further established by western blot analysis using rabbit polyclonal anti-human IL-lra (Abcam, USA). Protein concentration was determined spectrophotometrically by measuring absorbance at 280 nm and calculated using the following equation:

$$\boldsymbol{A_{280} = \varepsilon c l}$$

where, $\boldsymbol{\varepsilon}$ is molar extinction coefficient l/(mol × cm), $\boldsymbol{A}$ is absorbance at 280 nm, $\boldsymbol{c}$ is concentration (mol/l), $\boldsymbol{l}$ is the path length of cuvette (cm), $\boldsymbol{\varepsilon}$ for IL-1ra, IL-1ra1, IL-1ra2, IL-1ra3 = 15720 l/(mol × cm).

2.2. IL-1ra aggregation

IL-1ra was aggregated as described previously [19]. Briefly, 20 mg/ml of purified IL-1ra in citrate sodium EDTA buffer (CSE; 10 mM; pH 6.5) was incubated at 47 °C for 2–4 h and turbidity was measured at 405 nm on a UV-vis spectrophotometer.

2.3. Multimerisation assay

Multimerisation of IL-1ra, IL-lra1 and IL-lra3 was carried out under isothermal conditions; l ml of 20 mg/ml IL-1ra, IL-lra1 or IL-lra3 in 50 mM sodium phosphate buffer pH 6.0 was aliquoted into a 2 ml microcentrifuge tube and incubated at 37 °C and 200 rpm. Kinetics of multimerisation was followed by measuring turbidity (405 nm) at every 30 min interval on a UV-vis spectrophotometer.

2.4. Thioflavin T (ThT) binding assay

For Thioflavin T (ThT) binding assay 10 μl of samples collected were incubated with 190 μl of ThT (50 μM, Sigma) for 15 min in dark at 25 °C. Thereafter, fluorescence spectra were acquired on a spectrofluorimeter (Jobin Yvon Fluoromax) using an excitation and emission slit width of 5 nm. Samples were excited at 420 nm and emission was recorded in the range of 450–600 nm.

2.5. Congo red (CR) binding assay

For CR binding assay, 10 μl of samples drawn were incubated with 190 μ1 of Congo red dye (50 μM; Sigma) at 37 °C for 1 h in dark. Subsequently, absorption spectrum from 400 to 600 nm was acquired on UV-vis spectrophotometer (Shimadzu).

2.6. Atomic force microscopy

Pico plus Atomic Force Microscope (Agilent Technologies) was used in non-contact mode for imaging. Multimers from samples drawn at various time points were harvested by centrifugation at 10,000 rpm for 10 min at 4 °C. The resulting pellet was resuspended in 100 μl water and immobilized on freshly cleaved mica for 2 min. Samples were washed with ultrapure water, dried and subjected to AFM analysis.

2.7. Proteinase K digestion assay

Proteinase K digestion assay was performed as described previously [18]. Briefly, 200 μl of 20 mg/ml of IL-1ra1 or multimeric IL-1ra1 was incubated with 1:2000 or 1:5000 times diluted proteinase K (2 mg/ml) at 37 °C. Samples were withdrawn at regular intervals *viz.* 0, 5, 10, 15, 30 and 45 min, electrophoresed on 15% SDS-PAGE and stained with coomassie brilliant blue (CBB).

2.8. In-vitro release assay

Multimers of $O.D._{405}$ 1.6–2.0 were harvested, washed and incubated in 5 ml of phosphate buffered saline (PBS, pH 7.4) at 37 °C with slow rotation. Aliquots withdrawn at regular intervals (0, 3, 6, 9, 12, 15, 18, 21, 24, 48, 72, 96, 120 and 144 h) were centrifuged (10,000 rpm, 10 min) and absorbance of the supernatant was measured at 280 nm. Aliquot withdrawn each time was replaced with equal volume of PBS.

2.9. Assay for biological activity: D10.G4.1 proliferation

D10.G4.1 cell line (a murine IL-1 responsive helper T-cell line (TIB-224), purchased from American type tissue culture collection (ATCC) was cultured and maintained as per supplier's protocol. Biological activity of IL-lra or its variants or released monomers was assayed as described previously [20]. Briefly, D10.G4.1 cells growing logarithmically in RPMI 1640 (Sigma) containing 10% FBS (Gibco), 5×10^{-5} M β-mercaptoethanol (β-ME, Sigma) and 2 μg/ml Concanavalin A (ConA, Sigma) were plated into microtiter tissue culture plates flat bottom (2×10^4 cells/well), treated with IL-1ra, its variants or released monomers (15 ng/ml) 1 h prior to addition of mouse IL-1β (0.15 ng/ml, Sigma, USA) and 48 h later cells were pulsed with 0.5 μCi of [^{3}H] thymidine (Perkin Elmer). After 18 h cells were harvested onto glass fiber filters (Filter mat Type A, Wallac) and radioactivity incorporated was measured in a liquid scintillation counter (Perkin Elmer).

2.10. Assay for biological activity: TNF-α production by SW-982 cells

SW-982 cells (a human synovial cell line SW-982, HTB-93) procured from ATCC was maintained as per provider's instructions. For *in vitro* biological assays, SW-982 cells were plated at a density of 10^5 cells per well in a 24-well tissue culture plate, after 48 h cells were rinsed thoroughly with PBS, treated with 0.05 (10×) and 0.5 μg/ml (100×) of IL-1ra, its variants or released monomers and 1 h later stimulated with 5 ng/ml of human IL-1β (Sigma). After 24 h culture supernatants were analysed for human TNF-α levels using Ready Set Go® ELISA kits from eBioscience as per manufacturer's instructions.

2.11. IL-1 receptor binding assay

Flat bottom maxisorp plates (Nunc) were coated with 0.1 mg/ml (100 μl) of soluble human IL-1 receptor type I (Sigma), reconstituted in PBS pH 7.4, by incubating overnight at 4 °C. Next morning, the plates were washed thoroughly with PBS and blocking was done with 1% bovine serum albumin (BSA, 200 μl) at room temperature for 2 h. Subsequent to blocking two fold serial dilutions of IL-1ra, IL-1ra1 or released monomers of IL-1ra1 (rmIL-1ra1; 250–7.8 μg/ml) were added in triplicates (100 μl/well) and incubated for 1 h at room temperature. After washing thoroughly with PBS, rabbit anti-human IL-1ra (100 μl, 1:500 diluted in PBS containing BSA Abcam) was added to the wells and incubated for 1 h at room temperature. After washing thoroughly with PBST (PBS plus 0.01% Tween-20) HRP conjugated secondary antibody (100 μl, 1:3000 diluted in PBST containing 1% BSA, Santa Cruz Biotechnology, USA) was added for 30 min at room temperature. Thereafter, wells were washed thoroughly with PBST and incubated with substrate 3,3′,5,5′-Tetramethylbenzidine (100 μl, TMB, Sigma). On colour development reaction was stopped by adding 2 N sulfuric acid, 50 μl/well. Absorbance was measured at 450 nm and reference O.D. at 570 nm was subtracted from all readings.

2.12. Intrinsic fluorescence assay

0.1 mg/ml of IL-1ra, IL-1ra1, rmIL-1ra1 were excited at 280 nm and emission spectra between 300-500 nm was recorded for tryptophan fluorescence in a spectrofluorimeter (Jobin Yvon Fluoromax).

2.13. Mice

DBA/1J mice procured from Jackson Laboratories were housed and bred under standard conditions with *ad libitum* access to food and water. Animals were allowed