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Development and Characterization of a Fusion Protein Between Thermally Responsive Elastin-like Polypeptide and Interleukin-1 Receptor Antagonist

Sustained Release of a Local Antiinflammatory Therapeutic

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Objective. **Interleukin-1 receptor antagonist (IL-1Ra) has been evaluated for the intraarticular treatment of osteoarthritis. Such administration of proteins may have limited utility because of their rapid clearance and short half-life in the joint. The fusion of a drug to elastin-like polypeptides (ELPs) promotes the formation of aggregating particles that form a "drug depot" at physiologic temperatures, a phenomenon intended to prolong the presence of the drug. The purpose of this study was to develop an injectable drug depot composed of IL-1Ra and ELP domains and to evaluate the properties and bioactivity of the recombinant ELP-IL-1Ra fusion protein.**

Methods. **Fusion proteins between IL-1Ra and 2 distinct sequences and molecular weights of ELP were overexpressed in *Escherichia coli*. Environmental sensitivity was demonstrated by turbidity and dynamic light scattering as a function of temperature. IL-1Ra domain activity was evaluated by surface plasmon resonance, and in vitro antagonism of IL-1–mediated lymphocyte and thymocyte proliferation, as well as IL-1–induced tumor necrosis factor α (TNFα) expression and matrix metalloproteinase 3 (MMP-3) and ADAMTS-4 messenger RNA expression in human intervertebral disc fibrochondrocytes. IL-1Ra immunoreactivity was assessed before and after proteolytic degradation of the ELP partner.**

Results. **Both fusion proteins underwent supramolecular aggregation at subphysiologic temperatures and slowly resolubilized at 37°C. Interaction with IL-1 receptor was slower in association but equivalent in dissociation as compared with the commercial antagonist. Anti–IL-1 activity was demonstrated by inhibition of lymphocyte and thymocyte proliferation and by decreased TNFα expression and ADAMTS-4 and MMP-3 transcription by fibrochondrocytes. ELP domain proteolysis liberated a peptide of comparable size and immunoreactivity as the commercial IL-1Ra. This peptide was more bioactive against lymphocyte proliferation, nearly equivalent to the commercial antagonist.**

Conclusion. **The ELP-IL-1Ra fusion protein proved to retain the characteristic ELP inverse phase-transitioning behavior as well as the bioactivity of the IL-1Ra domain. This technology represents a novel drug carrier designed to prolong the presence of bioactive peptides following intraarticular delivery.**

Interleukin-1 (IL-1) has been implicated as a mediator of anabolic and catabolic processes in the progression of osteoarthritis (OA). IL-1 is a 17-kd protein that stimulates both synoviocytes and chondrocytes to secrete proteases and other mediators of inflam-

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mation (1). Enhanced production of IL-1 messenger RNA (mRNA) (2) and protein (3), and biologically relevant levels of this mediator have been found in the synovial fluid (4) and tissues of OA patients. This molecule creates a catabolic milieu and promotes chondrocyte apoptosis (5) and cartilage degradation (6), impedes the de novo synthesis of types II and IX collagen by the extracellular matrix (7), and alters the production of sulfated glycosaminoglycans (8,9).

Signaling through the IL-1 receptor is modulated by another binding protein, IL-1 receptor antagonist (IL-1Ra), which competitively antagonizes the binding of IL-1. Recombinant human IL-1Ra protein expressed in *Escherichia coli* retains its ability to antagonize IL-1–induced proliferation of several cell lines (10), downregulates the activity of matrix metalloproteinases (MMPs), and promotes the synthesis of collagen and glycosaminoglycans in cartilage explants (11). Recombinant IL-1Ra has been approved as treatment for rheumatoid arthritis (RA) and has been shown to slow cartilage degradation and to provide relief from joint symptoms when administered subcutaneously (5,12). However, systemic delivery of IL-1Ra (100 mg daily) exposes patients to complications that include injection site reactions, serious bacterial infection, particularly in asthmatic patients, and neutropenia (13). These toxic effects may be minimized by delivering the IL-1Ra protein from a local drug depot that provides for sustained release and attenuated peak serum exposure.

Local delivery of IL-1Ra into the joint space has been evaluated in a canine model of OA, with twice weekly intraarticular injections of 2 mg or 4 mg of protein being found to be chondroprotective (14). The safety of intraarticular IL-1Ra delivery has also been demonstrated in human clinical trials using single injections of 150 mg (15). As an alternative to the high and frequent dosing required for intraarticular protein delivery, animal studies have investigated IL-1Ra gene delivery via injection of either gene (16) or transfected cells (17), and exploratory clinical trials of such gene therapy have been performed in RA patients (18) and in OA patients (19).

The objective of this study was to develop an injectable drug depot composed of IL-1Ra and elastin-like polypeptide (ELP) domains. ELPs are genetically engineered polypentapeptide biopolymers with structural homology to mammalian elastin. They are composed of pentapeptide repeats of Val-Pro-Gly-X-Gly, where the guest residue is any amino acid residue other than proline (20). These compounds are soluble below their characteristic transition temperature and undergo an abrupt inverse temperature phase transition to form micron-sized multiparticle aggregates upon heating.

Previous studies have demonstrated that an ELP designed to undergo thermal phase transition upon intraarticular injection exhibited a 25-fold increase in its intraarticular half-life as compared with a soluble, nontransitioning ELP (21). Furthermore, systemic exposure to the injected ELP was substantially decreased by the phase-transitioning property of the polymer (21). In a separate study, local hyperthermia was shown to trigger ELP phase transitioning in solid tumors following systemic injection, promoting the formation of a drug depot that is anticipated to both heighten and prolong exposure to the attached drug (22). These results suggested that the inverse phase transition behavior of an ELP conjugated to a drug provides a potentially facile and valuable means of generating an intraarticular drug depot through direct injection into the joint space, allowing for slow resolubilization from the depot that could increase the drug longevity in the targeted compartment (23).

ELPs have been chemically conjugated to doxorubicin and have been shown to retain thermally induced aggregating properties after drug coupling, as well as comparable in vitro cytotoxicity against the squamous cell carcinoma line FaDu (24). Biologic activity was also retained by ELP fused to Penetratin, which underwent effective membrane translocation, as well as by ELP fused to H1, a c-*myc* oncogene inhibitor, which antagonized the transcriptional activation and retarded the growth of the breast carcinoma cell line MCF-7 (25). In addition, surfaces coated with an ELP fused to the RGD or fibronectin CS5 cell-binding sequence retained the ability to promote endothelial cell adhesion and spreading in vitro (26).

Attractive features of the recombinant ELP fusion protein technology include this demonstrated ability to maintain both phase-transitioning behaviors of the ELP as well as the activity of the drug domain, high-yield expression of chimeric proteins (27), and high drug-to-carrier ratios as compared with potentially suboptimal chemical conjugation. Furthermore, multiple studies have demonstrated the nonimmunogenicity, nonpyrogenicity, and biocompatibility of the ELP sequences with endogenous amino acid degradation products (28). Other attractive features of ELP fusion proteins include precise control over stereochemistry, polymer sequence, chain length (29,30), and ELP phase-transitioning temperature afforded by the recombinant protein synthesis (20,30).

The hypothesis of this study was that a fusion

protein constructed from ELP and IL-1Ra would retain the characteristic ELP inverse phase-transitioning behavior as well as the bioactivity of the IL-1Ra domain. An ELP-IL-1Ra gene was designed, and the protein product was expressed in *E coli*. Results are presented for the phase-transitioning and aggregating behaviors of the fusion protein, as well as the fusion protein IL-1 receptor binding affinity and in vitro bioactivity. The data demonstrate that ELP-IL-1Ra retains the functionality of both domains and that the bioactivity of the IL-1Ra domain is further enhanced when liberated by proteolysis from the ELP domain.

MATERIALS AND METHODS

Synthesis of fusion proteins. The human IL-1Ra gene was cloned from activated human monocyte-like histiocytic lymphoma cells (U937) with 100 μg/ml of lipopolysaccharide (31). RNA was isolated and complementary DNA (cDNA) was generated by reverse transcription–polymerase chain reaction (RT-PCR). The IL-1Ra–coding region was amplified by RT-PCR using the primers CGGGATCCGGGAGAAAATCCAGCAAGATG (forward) and CGGAATTCCCCTACTCGTCCTCCTGGA (reverse). These primers were designed to exclude the signal sequence for protein export to the extracellular space and to introduce 2 different restriction enzyme sites, a 5′ *Bam* HI and a 3′ *Eco* RI, for the purpose of directional and in-frame ligation to the ELP gene. Both deletion of the leader sequence (32,33) and insertion of restriction enzyme sites (34) have previously been performed to synthesize human recombinant IL-1Ra, and neither the bioactivity nor other functionality of the resulting protein was altered. The resultant cDNA was ligated into an *Srf* I linearized pPCR-Script Amp SK(+) cloning vector and transformed into XL10-Gold Kan ultracompetent *E coli* cells (both from Stratagene, La Jolla, CA). An IL-1Ra gene cassette was excised from this plasmid using *Bam* HI and *Eco* RI restriction enzymes and was purified by agarose gel extraction after electrophoresis.

A pET25b(+) expression vector was modified to contain *Sfi* I, *Bam* HI, and *Eco* RI restriction sites by ligating custom-designed oligonucleotides (Integrated DNA Technologies, Coralville, IA) into *Nde* I and *Eco* RI sites. The IL-1Ra gene cassette was ligated into this modified vector using *Bam* HI and *Eco* RI sites. The 2 chosen ELP sequences were designated ELP-$(V_5G_3A_2)_{90}$ (guest residue is Val-Gly-Ala in a 5:3:2 ratio; 90 pentapeptides) and ELP-$(V)_{30}$ (guest residue is Val; 30 pentapeptides). Corresponding genes were excised from previously constructed pUC19 cloning vectors by digestion with *Pfl* MI and *Bgl* I (30), purified by agarose gel extraction after electrophoresis, and subcloned into an *Sfi* I linearized IL-1Ra–containing pET25b(+) vector. Expression vectors containing the fusion gene were transformed into *E coli* BLR(DE3) (Novagen, Madison, WI) for protein hyperexpression (35).

One liter of TB Dry medium (Mo Bio, Carlsbad, CA) with 100 μg/ml of ampicillin was inoculated with the expression strain and grown using a hyperexpression protocol. Cells were harvested by centrifugation (3,200*g* for 15 minutes) and resuspended in 50 ml of phosphate buffered saline (PBS). Cells were lysed by sonication at 4°C and centrifuged (11,500*g* for 15 minutes, 4°C) to eliminate cell debris. Nucleic acids were precipitated using polyethyleneimine (0.5% weight/volume) and removed by centrifugation (11,500*g* for 15 minutes at 4°C). Protein was purified by 3 rounds of inverse transition cycling (ITC) as described previously (29,36).

Characterization of fusion proteins. The phase-transitioning behavior of the fusion proteins was characterized by ultraviolet-visible (UV-vis) spectrophotometry and by dynamic light scattering (DLS). The optical density at 350 nm (OD_{350}) of 500 μM fusion protein solutions in PBS was measured in the 15–60°C range (Cary 300 UV-vis spectrophotometer with multicell thermoelectric temperature controller; Varian, Walnut Creek, CA) to determine the transition temperature, which is defined as the temperature at which the OD_{350} is increased by 5% of the maximum observed change. The temperature-dependence of the hydrodynamic radius of the soluble ELP fusion protein and its aggregates were determined for a 25 μM solution in PBS filtered through a 20-nm Whatman Anodisc filter by DLS (90° scattering angle using a DynaPro LSR with Peltier temperature control; Wyatt Technology, Santa Barbara, CA). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed at 80 mV with ReadyGel 4–20% gradient gels (Bio-Rad, Hercules, CA), and was stained with SimplyBlue (Invitrogen, Carlsbad, CA) to confirm the purity of fusion protein solutions. Concentrations were determined spectrophotometrically using calculated extinction coefficients as described by Gill and von Hippel (37).

In vitro degradation of fusion proteins. Collagenase I or collagenase II (Sigma-Aldrich, St. Louis, MO) was dissolved in PBS to a concentration of 80 units/ml. Five microliters of the enzyme solution was added to 100 μl of a 100 μM ELP-IL-1Ra solution in PBS for a final activity of 0.4 units. The mixture was incubated with gentle agitation for 36 hours at 37°C, after which SDS-PAGE was performed as described above.

IL-1 receptor binding affinity of ELP-IL-1Ra. Surface plasmon resonance (SPR) analysis of the binding interaction between ELP-IL-1Ra and the IL-1 receptor was performed with a BIAcore X instrument (BIAcore, Uppsala, Sweden). Soluble IL-1 receptor type I (50 μl at a concentration of 1.67 μM in PBS) was immobilized onto a self-assembled monolayer of 16-mercaptohexadecanoic acid on gold-coated glass slides using activated amine chemistry. Ten microliters of an equal volume mixture of 0.2*M* *N*-hydroxysulfosuccinimide and 0.4*M* 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide was added to activate the terminal carboxylic acid groups of the self-assembled monolayer, after which the receptor was added. Thirty-five microliters of 0.2% bovine serum albumin was used to deactivate residual carboxylic acid groups, after which the surface was washed with PBS (5 μl/minute for 3 hours).

We measured the binding kinetics at room temperature by monitoring the changes in the refractive index of the surface upon injection of 15-μl aliquots of the test solution (500 nM solutions in PBS) at a flow rate of 5 μl/minute: ELP-IL-1Ra fusion proteins, commercial IL-1Ra (R&D Systems, Minneapolis, MN), or free ELP. We measured the dissociation kinetics by changing the overlying flow to PBS. Using nonlinear least-squares regression, the data were fit to a

model of bimolecular interaction (BIAevaluation Software; BIAcore). Five replicates of the SPR binding kinetics were performed for each protein, and the results for the association rate constant (k_{on}), the dissociation rate constant (k_{off}), and the equilibrium dissociation constant (K_D) were analyzed by a one-factor ANOVA with the post hoc Student-Newman-Keuls test for differences among the 2 fusion proteins, the commercial IL-1Ra, and the free ELP at a significance level of $\alpha = 0.05$.

In vitro disaggregation of fusion proteins. Solutions of ELP or ELP-IL-1Ra at a concentration of 25 mg/ml were thermally aggregated in a volume of 1.5 ml at the bottom of 15-ml conical tubes by heating to 37°C followed by warm centrifugation. Proteins were analyzed in triplicate. Aliquots (7–10 μl) were periodically taken, and concentrations were measured by UV-vis spectrophotometry until the aqueous phase concentration was observed to reach steady-state. The entire supernatant was withdrawn and replaced with an equivalent volume of PBS at 37°C. Aqueous-phase aliquots (7–10 μl) were withdrawn intermittently and concentrations measured by UV-vis spectrophotometry to monitor protein release into the supernatant.

Data were transformed into the mass fraction of ELP in the soluble form, and then fit by nonlinear regression to a first-order dissolution equation (equation 1):

$$m^*(t) = m^*_{SS}\,(1 - e^{-t/\tau})$$

where $m^*(t)$ is the mass fraction of the soluble form of ELP as a function of time, m^*_{SS} is the steady-state mass fraction of the soluble form ELP, and τ is the characteristic time constant. Goodness of fit was evaluated by the coefficient of determination (r^2). Single-factor ANOVA with post hoc Dunn's test was used to assess differences in steady-state ELP partition and characteristic time between the 2 fusion proteins and the free ELP at a significance level of 0.05.

IL-1Ra immunoreactivity of fusion protein and degradation products. A sandwich enzyme-linked immunosorbent assay (ELISA) (Quantikine Human IL-1Ra ELISA kit DRA00; R&D Systems) was used to quantify the immunoreactivity and accessibility of the ELP-IL-1Ra fusion protein and its degradation products at room temperature relative to a commercial recombinant human IL-1Ra. Student's *t*-test for samples of unequal variance was used to compare the immunoreactivity of each fusion protein against that of the commercial IL-1Ra (R&D Systems) and the negative control (nonfusion ELP) at a significance level of 0.05.

IL-1 antagonist bioassay of fusion proteins and degradation products using RPMI 1788 lymphocytes. The human peripheral blood lymphocyte line RPMI 1788 cells (American Type Culture Collection, Rockville, MD) were grown in suspension in 75-cm^2 tissue culture flasks containing RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 m*M* L-glutamine, 1 m*M* sodium pyruvate, 50 μ*M* β-mercaptoethanol, 10 m*M* HEPES buffer, 50 units/ml of penicillin, and 50 μg/ml of streptomycin. The cells were suspended in 80 μl of assay medium (culture medium with 3% heat-inactivated FBS) in a 96-well plate at a density of 1,000 cells/well and incubated at 37°C for 48 hours. Cells were stimulated with 29 p*M* IL-1β and treated with an antagonist (ELP-IL-1Ra, commercial IL-1Ra, digested ELP-IL-1Ra fragments, or nonfusion ELP) at concentrations between 0 and 500 molar excess above that of IL-1β. The assay plate was incubated for 48 hours, after which cell proliferation was quantified by the CellTiter Glo luminescence assay (Promega, Madison, WI). Treatment effects were normalized against the observed proliferation of IL-1β–stimulated controls.

The dose-response relationship was fitted by nonlinear regression to a logistic curve to derive the 50% inhibition concentration (IC_{50}) data, using the following equation (equation 2):

$$\%I = \%I_{Max} + \frac{(\%I_{Baseline} - \%I_{Max})}{\left(1 + \frac{[\text{antagonist}]}{IC_{50}}\right)^k}$$

where %I is the concentration-dependent fractional inhibition of IL-1β–induced proliferation by a given antagonist, $\%I_{Max}$ is the maximal observed antagonism of proliferation, and $\%I_{Baseline}$ is the observed proliferation without antagonist. The hill slope (k) and the inhibitory concentration of the antagonist (IC_{50}) are fit parameters. Goodness of fit was evaluated by the coefficient of determination (r^2). Single-factor ANOVA with post hoc Dunn's test evaluated differences among the antagonist IC_{50} values at a significance level of 0.05.

IL-1 antagonist bioassay of fusion proteins using murine thymocytes. Murine thymocytes were mechanically isolated from freshly killed C57BL/6 mice and grown in suspension in 75-cm^2 tissue culture flasks containing RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum, 1 μg/ml of concanavalin A (Sigma-Aldrich), and 1 ng/ml of IL-1β. The cells were incubated at a density of 2×10^6 cells/200 μl, and the media were treated with either the commercial IL-1Ra or the ELP-$(V)_{30}$-IL-1Ra fusion protein for 48 hours. The culture was pulsed with tritiated thymidine (0.5 μCi/well; PerkinElmer, Boston, MA) 6 hours prior to harvesting. The cells were then harvested onto a glass-fiber filter and dried in a microwave. Incorporated radioactivity was then determined by liquid scintillation counting in a Microbeta 1450 Trilux scintillation counter (PerkinElmer). Antagonism of thymocyte proliferation was observed by the dose-dependent inhibition of cellular thymidine incorporation. As described above, the dose-response relationship was fitted to equation 2, and differences in the IC_{50} were evaluated.

IL-1 antagonist bioassay of fusion proteins using human intervertebral disc cells. Fibrochondrocytic cells were isolated from the anulus fibrosus and nucleus pulposus regions of human intervertebral discs obtained from cadavers (Thompson degenerative grades 2–3 [38]). This involved sharp dissection of the anulus fibrosus, blunt separation of the nucleus pulposus, and sequential enzymatic digestion of both tissues with 0.4% Pronase (EMD Bioscience, La Jolla, CA) for 1 hour, followed by 0.025% collagenase P (Roche Applied Science, Indianapolis, IN) and 0.001% DNase II (Sigma-Aldrich) for 16 hours at 37°C in an atmosphere containing 5% CO_2. Cells were then cultured in 1.2% alginate to maintain a rounded morphology (Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 10% FBS, 25 μg/ml of ascorbic acid, 360 μg/ml of L-glutamine, and 50 μg/ml of gentamicin). After 7 days of culture, cells were serum-starved for 24 hours, and then cultures containing 9 beads per well in a 24-well plate were treated with 275 p*M* IL-1β alone or were preincubated

with commercial IL-1Ra at 2 or 17 molar equivalents (corresponding to 590 p*M* or 4,722 p*M*, respectively) or with ELP-$(V)_{30}$-IL-1Ra fusion protein at 15 or 29 molar equivalents (corresponding to 4,000 p*M* or 8,000 p*M*, respectively). Following the 24-hour preincubation period, cells were cultured with IL-1β (275 p*M*) for an additional 24 hours.

After the incubation period, the media were collected to quantify tumor necrosis factor α (TNFα) expression with the use of an ELISA kit (BioSource, Carlsbad, CA). The beads were collected for quantitative PCR analysis of the total RNA extracted using primers specific for MMP-3 and ADAMTS-4 and GAPDH (internal control). A single-factor ANOVA with post hoc Fisher's least significant difference test was used to detect differences among the commercial and fusion protein antagonists at all concentrations evaluated.

RESULTS

Analysis of the synthesized fusion proteins. Human IL-1Ra was successfully cloned into the modified pET25b(+) expression vector, with subsequent adjacent insertion of ELP genes encoding ELP-$(V)_{30}$ (molecular weight 14 kd) or ELP-$(V_5G_3A_2)_{90}$ (molecular weight 37 kd) at the protein N-terminus. Both fusion proteins were successfully expressed in *E coli* BLR(DE3), with purification by ITC (23,36). SDS-PAGE revealed appropriate molecular weight bands for each protein, with minimal contamination, after 3 rounds of ITC (Figure 1A). The protein yield after such purification was 120–150 mg per liter of *E coli*.

Fusion protein characteristics. Both ELP-IL-1Ra fusion proteins exhibited phase-transitioning behavior based on the findings of the optical density and DLS experiments. Figure 2A shows the temperature dependence of optical density at 350 nm, with an increase in the amount of light scattered at temperatures above 34°C for ELP-$(V_5G_3A_2)_{90}$-IL-1Ra and above 32°C for ELP-$(V)_{30}$-IL-1Ra. The former protein has a broad transition, whereas the latter one has the more characteristic abrupt change of other ELP fusion proteins (39,40). The breadth of transition of the former protein may arise from affected folding and disulfide bonding patterns caused by the larger ELP-$(V_5G_3A_2)_{90}$ as compared with ELP-$(V)_{30}$. Evidence of a temperature-dependent increase in hydrodynamic radius from monomers to larger nanoparticles is shown by DLS of both fusion proteins (Figure 2B). In both cases, the particle sizes are consistently smaller than 5 nm for temperatures below the transition temperature, consistent with the presence of soluble protein monomers. Upon reaching the transition temperature, the proteins aggregate and the particle size increases dramatically, growing to the order of microns.

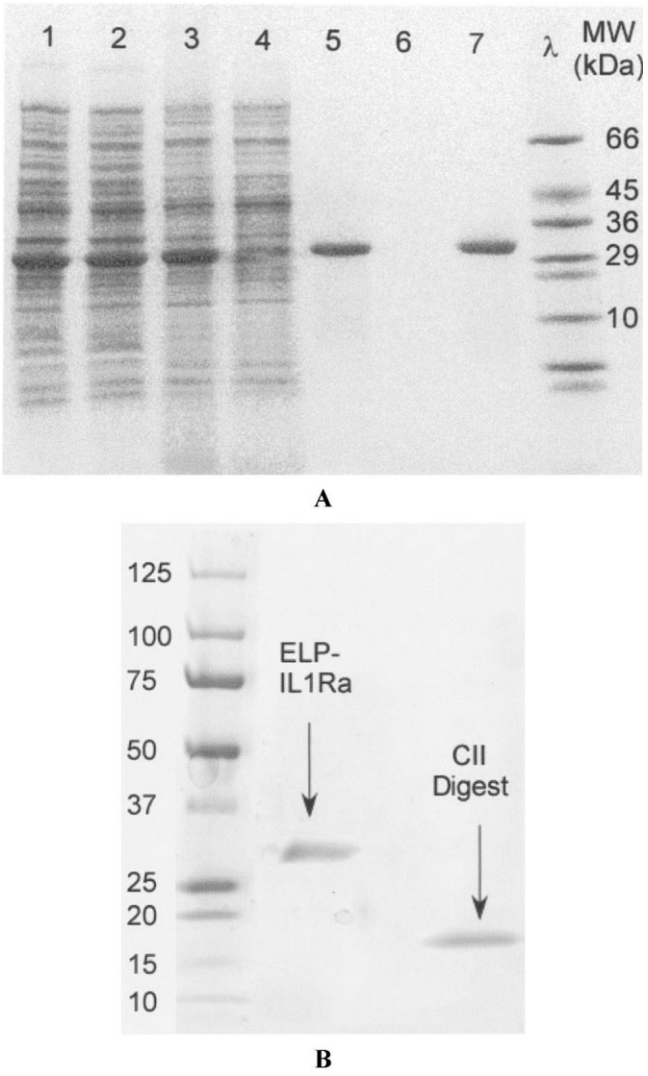

Figure 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analyses of the ELP-$(V)_{30}$-IL-1Ra fusion protein. **A,** SDS-PAGE analysis of ELP-$(V)_{30}$-IL-1Ra fusion protein purification after inverse transition cycling. Lane 1, cell lysate; lane 2, soluble lysate; lane 3, soluble proteins following polyethyleneimine treatment; lane 4, insoluble proteins following polyethyleneimine treatment; lane 5, soluble proteins after cold spin (4°C); lane 6, soluble proteins after hot spin (30°C); lane 7, soluble proteins after second cold spin (4°C). Excellent purity from other *Escherichia coli* proteins is observed after 3 inverse transition cycles. Molecular weight markers are shown at the right. **B,** SDS-PAGE analysis of ELP-$(V)_{30}$-IL-1Ra degradation after treatment with collagenase II (CII). The enzyme was added at an activity of 0.4 units/ml to a 10 nM solution of ELP-$(V)_{30}$-IL-1Ra fusion protein in phosphate buffered saline. Digestion was performed at 37°C for 36 hours. The 31-kd fusion protein disappears, and a band of ~17 kd appears. Molecular weight markers are shown at the left.

In vitro fusion protein degradation. Incubation of the ELP-IL-1Ra fusion proteins with collagenase I or collagenase II led to complete disappearance of the

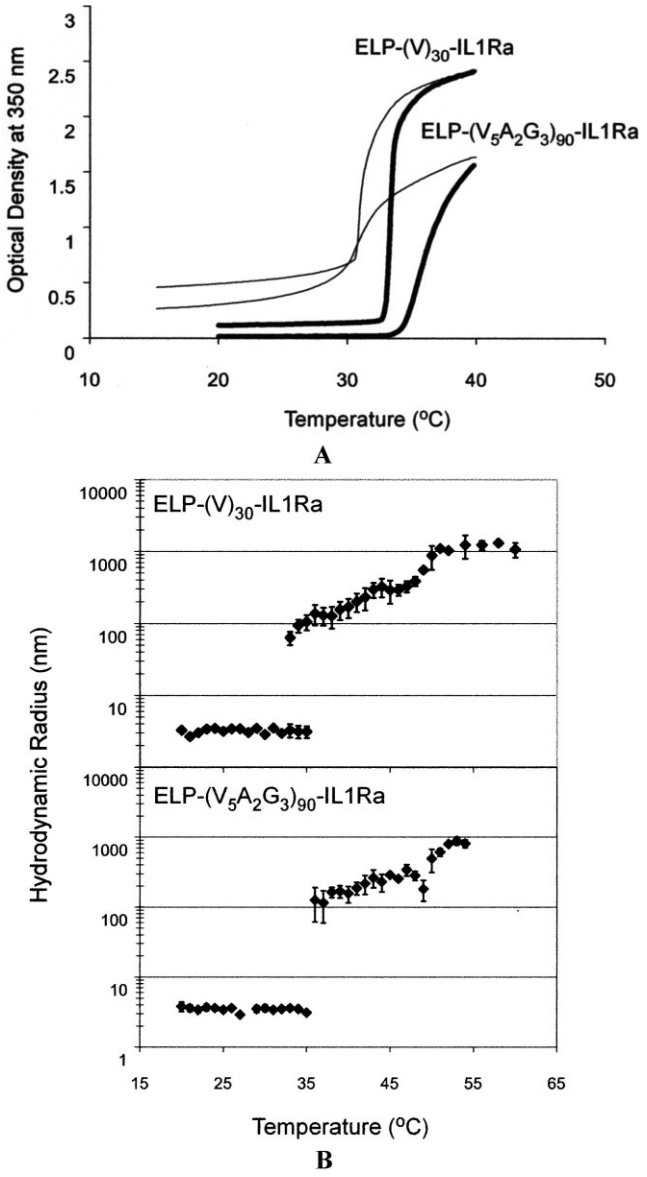

Figure 2. Characterization of ELP-IL-1Ra fusion proteins by optical density and dynamic light scattering analyses. **A,** Optical density of ELP-IL-1Ra fusion proteins as a function of temperature at 350 nm. A sharp phase-transition upon heating is observed for ELP-$(V)_{30}$-IL-1Ra at 34°C (thick line). In contrast, ELP-$(V_5G_3A_2)_{90}$-IL-1Ra undergoes a broad transition between 35°C and 40°C to aggregate upon reaching subphysiologic temperatures (thick line). Only partial reversibility is noted for both proteins (thin lines). **B,** Dynamic light scattering data (mean ± polydispersity) for ELP-$(V)_{30}$-IL-1Ra (top) and ELP-$(V_5G_3A_2)_{90}$-IL-1Ra (bottom) at 25 μM. A transition from monomers to larger, micron-sized aggregates at subphysiologic temperatures is shown.

fusion protein bands on SDS-PAGE (Figure 1B, showing only the results for collagenase II digestion) with the concomitant appearance of a new fragment of ~17 kd.

Analysis of IL-1 receptor binding affinity of ELP-IL-1Ra. SPR studies of fusion protein binding to the IL-1 receptor showed the K_D for both fusion proteins to be >10-fold higher than that of commercial IL-1Ra ($P < 0.05$) (Table 1). While the dissociation rate constant for the receptor-ligand complex (k_{off}) did not vary significantly between the fusion proteins and the commercial antagonist, the association rate constant (k_{on}) was significantly greater for the commercial IL-1Ra than for either of the fusion proteins ($P < 0.05$). The slower association for both fusion proteins relative to commercial IL-1Ra suggests that the ELP tag may sterically hinder their initial association with the IL-1 receptor, but the complexes formed between the IL-1 receptor and ELP-IL-1Ra fusion proteins are as stable as with commercial IL-1Ra.

In vitro fusion protein disaggregation. The time-dependent release of ELP-IL-1Ra fusion proteins and the ELP controls into fresh PBS supernatant is shown in Figure 3. The steady-state mass fraction of free ELP (m^*_{ss}) was determined to be 23.3 ± 0.9% (mean ± SD), with a time constant of 27 ± 4 hours (mean ± SD). Both ELP-$(V_5G_3A_2)_{90}$-IL-1Ra and ELP-$(V)_{30}$-IL-1Ra fusion proteins resolubilized to a greater extent, 34.5 ± 0.9% and 31.9 ± 1.0%, than did the ELP alone ($P < 0.05$). Also, both had shorter time constants of 19 ± 2 hours compared with free ELP, as expected for the fusion protein containing the more hydrophilic IL-1Ra domain at physiologic temperatures. The fusion proteins did not differ in their time constants of disaggregation, but a difference was noted in the steady-state mass fraction of free protein, with lesser release for the fusion protein with the larger molecular weight ELP tag ($P < 0.05$).

Analysis of IL-1Ra immunoreactivity of fusion proteins and degradation products. The ELP-$(V_5G_3A_2)_{90}$-IL-1Ra fusion protein was immunoreactive in the sandwich ELISA for IL-1Ra (Table 2), demonstrating authentic sequence and accessibility of the IL-1Ra domain to external antibody recognition despite the presence of the ELP partner. Nevertheless, the immunoreactivity was only 12.4 ± 0.4% (mean ± SD) of an equimolar solution of commercial recombinant human IL-1Ra, suggesting that the ELP may interfere with accessibility of the IL-1Ra epitope. Indeed, the 17-kd collagenase II–induced degradation fragment had significantly higher equimolar immunoreactivity (58 ± 4%; $P < 0.01$). Additionally, there was minimal nonspecific binding of ELP in the ELISA, with only 1.0 ± 0.5% relative immunoreactivity with 1,000 molar equivalents of IL-1Ra ($P < 0.01$). This result demonstrates the specificity of the ELISA for IL-1Ra domain of the fusion protein.

Table 1. Surface plasmon resonance data for the binding of IL-1 competitive antagonists to soluble IL-1 receptor*

Antagonist	k_{on}, $\times 10^4\ M^{-1} \cdot$ seconds^{-1}	k_{off}, $\times 10^{-4}$ seconds^{-1}	K_D, nM	$\frac{K_{D\ Antagonist}}{K_{D\ IL\text{-}1Ra}}$
IL-1Ra	41.9 ± 8.0	1.6 ± 0.5	0.4 ± 0.1	1.0
ELP-$(V_5G_3A_2)_{90}$-IL-1Ra	4.7 ± 0.2†	1.8 ± 0.3	3.8 ± 0.5†	9.6 ± 2.7
ELP-$(V)_{30}$-IL-1Ra	3.0 ± 0.3†	3.4 ± 1.1	13.5 ± 2.9†	33.8 ± 13.0
ELP-$(V)_{120}$	0.24†	63†	2.6 × 10^4†	6.5 × 10^4

* Values are the mean ± SD. IL-1 = interleukin-1; k_{on} = association rate constant; k_{off} = dissociation rate constant; K_D = equilibrium dissociation constant; IL-1Ra = IL-1 receptor antagonist; ELP = elastin-like polypeptide.
† $P < 0.05$ versus IL-1Ra.

IL-1 antagonist bioassay using RPMI 1788 lymphocytes. Figure 4 shows the bioactivity of recombinant human IL-1Ra and ELP-$(V_5G_3A_2)_{90}$-IL-1Ra and its collagenase digestion fragment against IL-1–induced lymphocyte proliferation. The commercial antagonist had an IC_{50} of 1.4 ± 0.3 nM (mean ± SD). Both fusion proteins were less active, with IC_{50} values of 730 ± 240 nM and 310 ± 120 nM for ELP-$(V_5G_3A_2)_{90}$-IL-1Ra and ELP-$(V)_{30}$-IL-1Ra, respectively ($P < 0.05$). However, collagenase digestion products of these 2 fusion proteins demonstrated significantly higher bioactivity than the parent fusion proteins, with an IC_{50} of 4.5 ± 1.5 nM for ELP-$(V_5G_3A_2)_{90}$-IL-1Ra and 5.0 ± 2.3 nM for ELP-$(V)_{30}$-IL-1Ra breakdown fragments ($P < 0.05$). These values for the bioactivity of ELP-$(V_5G_3A_2)_{90}$-IL-1Ra and ELP-$(V)_{30}$-IL-1Ra cleavage products were not significantly different from that of the commercial protein.

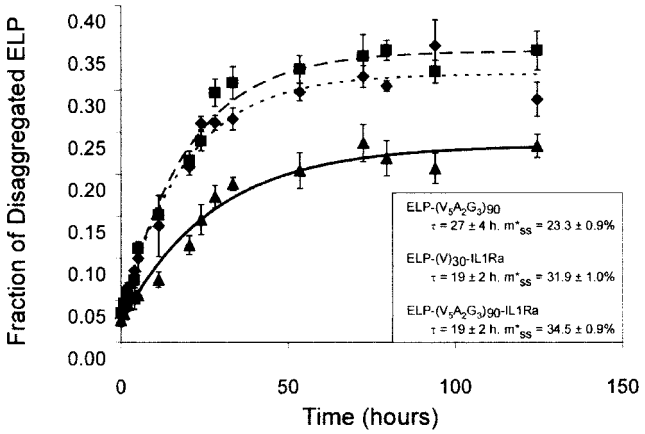

Figure 3. Disaggregation of fusion proteins in vitro. Elastin-like polypeptide (ELP) (▲) and ELP-IL-1Ra fusion proteins with $(V)_{30}$ (◆) and $(V_5G_3A_2)_{90}$ (■) tags disaggregate into warm phosphate buffered saline supernatants, reaching a steady-state thermodynamic equilibrium. Data were fit to an increasing monoexponential (see equation 1 in Materials and Methods), with coefficients of determination (r^2) ranging from 0.98 to 0.99. The extent of protein release into the supernatant was found to be greater for the fusion proteins than for the free ELP ($P < 0.05$), possibly because of the thermodynamic benefit conferred by fusion domain solvation that favors protein resolubilization from the aggregate. Furthermore, the ELP-IL-1Ra fusion proteins disaggregate more rapidly than do the nonfusion ELP molecules ($P < 0.05$).

IL-1 antagonist bioassay using murine thymocytes. Figure 5 shows the bioactivity of recombinant human IL-1Ra and ELP-$(V)_{30}$-IL-1Ra against IL-1–induced thymocyte proliferation. The commercial antagonist had an IC_{50} of 2.9 ± 1.1 nM. The fusion protein was active in inhibiting IL-1–dependent proliferation effects, although at significantly higher concentrations than that of the commercial IL-1Ra (IC_{50} 61 ± 19 nM; $P < 0.05$). This represents a more than 20-fold increase in the necessary concentration of the fusion protein than the free antagonist to elicit the same inhibitory response.

IL-1 antagonist bioassay using human intervertebral disc cells. Incubation of disc cells with IL-1β significantly up-regulated the expression of TNFα for cells of both the anulus fibrosus and the nucleus pulposus regions (Figure 6). Anulus fibrosus cells were more

Table 2. Immunoreactivity of ELP-$(V_5G_3A_2)_{90}$, ELP-$(V_5G_3A_2)_{90}$-IL-1Ra, and collagenase II degradation products of ELP-$(V_5G_3A_2)_{90}$-IL-1Ra*

Antagonist	IL-1Ra concentration	Fractional IL-1Ra immunoreactivity, %
ELP-$(V_5G_3A_2)_{90}$	1,000 molar equivalents	1.0 ± 0.5
ELP-$(V_5G_3A_2)_{90}$-IL-1Ra	Equimolar	12.4 ± 0.4†
Degradation fragment	Equimolar	58 ± 4‡

* Immunoreactivities were determined by sandwich enzyme-linked immunosorbent assay. Values are the mean ± SD. ELP = elastin-like polypeptide; IL-1Ra = interleukin-1 receptor antagonist.
† $P < 0.05$ versus ELP-$(V_5G_3A_2)_{90}$.
‡ $P < 0.05$ versus ELP-$(V_5G_3A_2)_{90}$-IL-1Ra.

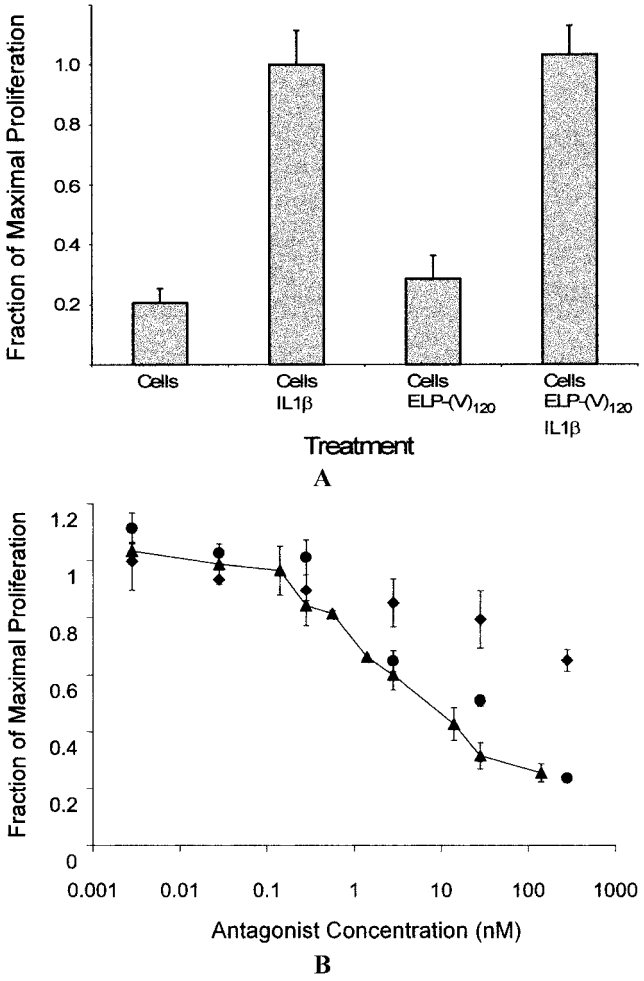

Figure 4. Interleukin-1 (IL-1) antagonist bioassays in RPMI 1788 lymphocytes. **A,** In vitro anti–IL-1 bioassay control experiments. RPMI 1788 lymphocytes incubated in culture media supplemented with ELP-$(V)_{120}$ did not show higher rates of proliferation than the controls in regular media ($P = 0.19$). Baseline rates were 21 ± 5% and 29 ± 8% of maximal proliferation for culture media controls and ELP-supplemented media, respectively (mean ± SD). Similarly, incubation with the same ELP did not affect the level of IL-1–induced lymphocyte proliferation ($P = 0.41$). Stimulated rates were 100 ± 11% and 103 ± 10% of maximal proliferation for IL-1–stimulated cultures and ELP-supplemented IL-1–stimulated cultures, respectively (mean ± SD). **B,** In vitro bioassay of fusion protein ELP-$(V_5G_3A_2)_{90}$-IL-1Ra. The bioactivity of ELP-$(V_5G_3A_2)_{90}$-IL-1Ra against IL-1–induced proliferation of RPMI 1788 lymphocytes is significantly reduced compared with recombinant human IL-1 receptor antagonist (IL-1Ra). The collagenase I digest of this chimeric protein (●) yields a fragment with significantly more bioactivity than the parent fusion protein (◆), approximating that of the commercial protein (▲). Coefficients of determination (r^2) for fitting to a dose-response curve (see equation 2 in Materials and Methods) ranged from 0.94 to 0.99. Values are the mean ± SD.

responsive to pretreatment with commercial IL-1Ra at the low concentrations, with nearly 50% inhibition of

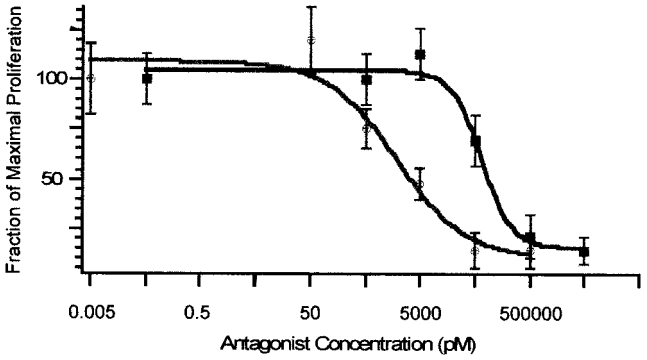

Figure 5. Interleukin-1 (IL-1) antagonist bioassays in murine thymocytes. In vitro bioactivity of ELP-$(V)_{30}$-IL-1Ra (■) against IL-1–induced murine thymocyte proliferation is significantly reduced compared with that of the recombinant human IL-1 receptor antagonist (IL-1Ra) (○). Coefficients of determination (r^2) for fitting to a dose-response curve (see equation 2 in Materials and Methods) ranged from 0.88 to 0.90. Values are the mean ± SD.

IL-1–dependent TNFα secretion. At higher IL-1Ra concentrations corresponding to 17 molar equivalents of IL-1β, both nucleus pulposus and anulus fibrosus cells

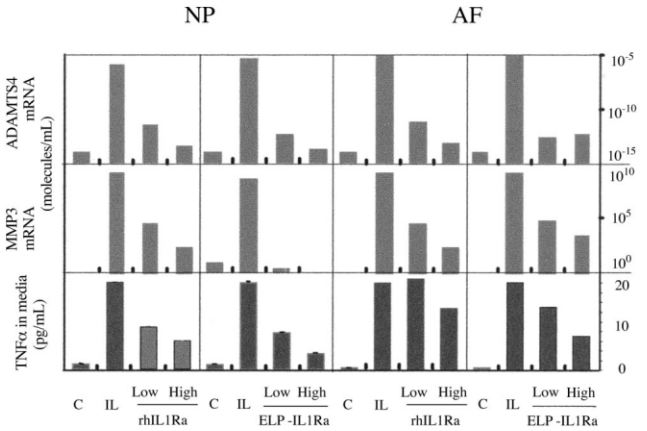

Figure 6. Interleukin-1 (IL-1) antagonist bioassays in human intervertebral disc cells. In vitro bioactivity of commercial IL-1 receptor antagonist (IL-1Ra) and ELP-$(V)_{30}$-IL-1Ra fusion protein against the IL-1–induced expression of tumor necrosis factor α (TNFα) and transcription of matrix metalloproteinase 3 (MMP-3) and ADAMTS-4 mRNA in nucleus pulposus (NP) cells and anulus fibrosus (AF) cells is shown. Cells were left untreated (control [C]) or were treated with 275 p*M* IL-1β (IL), or cells were pretreated for 24 hours with either a commercial recombinant human IL-1Ra (rhIL-1Ra) at low (2 equivalents) and high (17 equivalents) concentrations or the ELP-IL-1Ra fusion protein at low (15 equivalents) and high (29 equivalents) concentrations prior to incubation with added IL-1β for an additional 24 hours. Preincubation with the commercial or fusion protein antagonists led to substantial decreases in TNFα production, as determined by enzyme-linked immunosorbent assay ($P < 0.001$), as well as decreases in the levels of mRNA for MMP-3 ($P < 0.001$) and ADAMTS-4 ($P < 0.001$), in both nucleus pulposus and anulus fibrosus cells.

responded with attenuated levels of TNFα. Equivalent attenuation of TNFα levels was noted for pretreatment with the ELP-IL-1Ra fusion protein at concentrations of 15 and 20 molar equivalents in both cell types ($P < 0.001$), demonstrating nearly equivalent activity of the fusion protein with commercial IL-1Ra.

Treatment of both anulus fibrosus and nucleus pulposus cells with IL-1β also led to a dramatic up-regulation of levels of mRNA encoding MMP-3 and ADAMTS-4 (Figure 6), an effect that was attenuated by pretreatment with the commercial antagonist at both low and high concentrations ($P < 0.001$). The fusion protein at either concentration was similarly active in inhibiting IL-1β up-regulation of ADAMTS-4 mRNA in both cell types than was commercial IL-1Ra at 17 molar equivalents ($P < 0.001$). The fusion protein was exceptionally active in inhibiting the up-regulation of MMP-3 mRNA in nucleus pulposus cells, and was also active in inhibiting MMP-3 mRNA increases in anulus fibrosus cells ($P < 0.001$).

DISCUSSION

A fusion protein combining ELP and IL-1Ra was synthesized for local anti–IL-1 therapy in diarthrodial joints, designed to promote the in situ formation of a local IL-1Ra–containing drug depot via the thermal transitioning of the ELP domain upon intraarticular injection. In this study, 2 new proteins were expressed, consisting of an IL-1Ra protein fused to the C-terminus of the thermally responsive ELP domains ELP-$(V)_{30}$ and ELP-$(V_5G_3A_2)_{90}$. Both proteins exhibited thermal phase-transitioning behaviors of the ELP domains, with increased solution turbidity and hydrodynamic radii upon raising the solution temperature above 32°C and 34°C for the ELP-$(V)_{30}$ and ELP-$(V_5G_3A_2)_{90}$ domains, respectively.

The functionality of the ELP domain was further evaluated by studying the fusion protein disaggregation kinetics from an in vitro drug depot. Following the formation of a stable ELP aggregate, monomer or small-particle release was driven by disruption of thermodynamic equilibrium by replacing the supernatant with warm PBS. More extensive disaggregation was observed for the ELP-IL-1Ra fusion proteins than for ELP alone, possibly because of intrinsic IL-1Ra domain solubility. The release and reaggregation of monomers from the aggregate was assumed to occur by a first-order process, although it also remains possible that there were interactions in the overlying supernatant. Nevertheless, preservation of the aggregating and disaggregating behaviors of the fusion proteins indicates functionality of the ELP domain, a prerequisite for serving as an in vivo sustained-release drug delivery vehicle.

Both ELP-IL-1Ra molecules also retained functionality of the IL-1Ra domain. First, fusion protein binding to the IL-1 receptor was observed to occur, although with slower association constants (k_{on}) but similar dissociation constants (k_{off}) than the commercial antagonist. The difference in k_{on} values suggests that accessibility of ELP-IL-1Ra to the receptor is impaired in comparison to the commercial IL-1Ra, while the similar k_{off} values implies that the binding strength between the fusion proteins and the bound receptor are similar to that of the lone antagonist once the complex is formed. The consequently higher fusion protein K_D compared with the commercial IL-1Ra is anticipated because the added ELP domain is likely to impair the interaction of the IL-1Ra domain with its receptor.

Additional evidence of the functionality of IL-1Ra was provided by in vitro bioactivity assays, which demonstrated that both recombinant fusion proteins were capable of attenuating IL-1–dependent cell proliferation in an immortalized lymphocyte cell line and in primary thymocytes, although with less potency than that of the commercial IL-1Ra. In other assays of bioactivity, the ELP-$(V)_{30}$-IL-1Ra fusion protein was effective in attenuating the in vitro IL-1–dependent expression of TNFα as well as MMP-3 and ADAMTS-4 mRNA transcription in anulus fibrosus and nucleus pulposus disc cells. Last, the immunoreactivity of the ELP-$(V_5G_3A_2)_{90}$-IL-1Ra compound was only 12% of that seen for an equimolar quantity of free antagonist, providing another confirmation of the functionality of the IL-1Ra domain. It is also unlikely that the ELP domain is able to interact with elastin receptors, based on findings by Mecham and coworkers (41), who showed that a variety of hydrophobic sequences, including the repeat sequence VPGVG used here, do not interact with elastin receptors (with the exception of the hydrophobic hexapeptide cell recognition domain VGVAPG).

While both the K_D and IC_{50} values were not as favorable for the fusion protein as for the commercial antagonist, the necessary K_D or IC_{50} required for this protein to achieve drug efficacy will depend on the persistence of the drug in the joint, the total amount of protein delivered, the disaggregation kinetics, joint clearance, and more. Furthermore, caution should be exercised in making direct comparisons against the commercial recombinant human IL-1Ra because the gene sequence and technique for synthesis and purification will differ between the commercial drug and the

IL-1Ra domain in the fusion protein. The nature of these differences and the extent of their impact in limiting direct comparison are not well understood.

A surprising and significant finding of this study was that collagenase proteolysis of ELP-$(V_5G_3A_2)_{90}$-IL-1Ra yielded a 17-kd fragment with higher immunoreactivity and bioactivity than the parent fusion protein. Indeed, this fragment was only slightly less bioactive than the commercial antagonist. A similar fragment was also created by digestion of ELP-$(V)_{30}$-IL-1Ra. These findings together suggest that the attenuated bioactivity of the ELP-IL-1Ra fusion protein in inhibiting the effects of IL-1 is partly mediated by the unfavorable constraints imposed by the bulky ELP domain upon receptor–antagonist interactions. Release of this steric hindrance by ELP domain proteolysis enhanced both the immunoreactivity and the bioactivity without any further specific refolding. This observation reveals another advantage of the ELP tag: the IL-1Ra gene product may be purified by cyclic thermal transitioning of the fusion protein followed by enzymatic cleavage to yield a functional IL-1Ra protein (40). Previous studies have shown that trypsin-mediated degradation of cross-linked ELP hydrogels yields no discrete ELP fragments (42), suggesting that the cleaved ELP domain with identical periodic cleavage sites is completely degraded.

Collagenases are up-regulated in the arthritic joint (43), so in vivo enzymatic digestion may mediate the release of bioactive IL-1Ra at the pathologic site. This degradation may affect the intraarticular half-life of the delivered IL-1Ra domain, leading to more rapid clearance from the joint. We previously showed the feasibility of intraarticular delivery of ELP alone, as a low-viscosity, room-temperature solution that aggregates at physiologic temperatures to form a drug depot in the synovial joint space that is capable of sustained release and slow clearance from the joint (21). In these biodistribution studies, an aggregating, depot-forming ELP was associated with a joint half-life of 88 ± 5 hours, as compared with 3.4 ± 0.2 hours for a soluble ELP of comparable molecular weight (21). As such, the balance of competing digestion and distribution will ultimately govern the necessary dose for treating this arthritis.

Specific introduction of the thermally responsive ELP tag on the end of IL-1Ra, as shown here, is expected to yield high local doses when delivered via intraarticular injection, provide sustained release and lower systemic exposure, and ideally, reduce systemic side effects. In proposing the ELP fusion protein approach for treating monarticular diseases such as OA, several factors must be considered. While Urry and coworkers (28) have demonstrated biocompatibility and nonimmunogenicity for ELPs, the immunogenicity of the chimeric fusion protein remains unknown and would require in vivo testing. Furthermore, IL-1Ra as a specific target must also be considered, given the complex role played by IL-1 in regulating anabolic and catabolic processes in the joint. A previous study of IL-1–deficient mice demonstrated accelerated development of OA lesions following surgical transection of the medial collateral ligament and partial medial meniscectomy (44). Pathologic changes included greater severity of tibial plateau cartilage lesions and localized immunopositivity of MMP-, aggrecanase-, and collagenase-generated neoepitopes. This study does not suggest that IL-1 is cytoprotective, but rather, it illustrates the importance of a homeostatic balance between anabolic and catabolic cartilage processes in maintaining cartilage and joint health. In the absence of IL-1, it is possible that overcompensation by other proinflammatory mediators may complicate joint susceptibility to cartilage degradation.

In conclusion, genetic engineering of protein-based therapeutic agents has permitted the synthesis of a thermally responsive polypeptide coupled with a protein therapeutic, with the goal of combining the unique features of each constituent. The in vitro studies of functionality of the IL-1Ra domain and the ELP domain are necessary steps toward designing a thermally triggered drug-releasing depot in the joint space. Although attenuated bioactivity was observed for the ELP-IL-1Ra fusion proteins compared with the lone antagonist, this is tolerable if sustained protein release from a slowly disaggregating drug depot prolongs the presence of therapeutic drug levels in the joint space. The facile collagenase degradation of the ELP domain from the fusion protein is an important ancillary feature that may promote the therapeutic efficacy of this drug delivery system. Bioactive peptides fused with thermally responsive ELP domains may represent a novel drug delivery system that preserves bioactivity while introducing desirable environmental sensitivity. This could form a drug depot that exhibits sustained release into a target compartment, protecting against associated immunosuppressant toxicities of systemic delivery.

AUTHOR CONTRIBUTIONS

Drs. Setton and Shamji had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Shamji, Betre, Kraus, Chen, Chilkoti, Pichika, Masuda, Setton.

Acquisition of data. Shamji, Betre, Pichika.

Analysis and interpretation of data. Shamji, Betre, Kraus, Chen, Chilkoti, Pichika, Masuda, Setton.
Manuscript preparation. Shamji, Betre, Kraus, Chen, Chilkoti, Masuda, Setton.
Statistical analysis. Shamji, Betre, Setton.

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