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□ Original Article □

Inhibition of the IL-1β-induced Expression of Matrix Metalloproteinases by Controlled Release of IL-1 Receptor Antagonist Using Injectable and Thermo-reversible Gels in Human Osteoarthritis Chondrocytes

Jae-Bum Jun[1], Jang Kyoung Kim[2], Tae-Hwan Kim[1], Young-In Na[3], Choong Hyeok Choi[4], Yong-Hee Kim[2]

Department of Rheumatology, Hanyang University Hospital for Rheumatic Diseases[1], Department of Bioengineering, College of Engineering, Hanyang University[2], Institute of Rheumatism, Hanyang University[3], Department of Surgery for Rheumatism, Hanyang University Hospital for Rheumatic Diseases[4], Seoul, Korea

***Objective*. IL-1β is involved in the degradation of articular cartilage in various arthritides, including osteoarthritis (OA). Competitive inhibition of IL-1β by IL-1 receptor antagonists (IL-1Ra) may represent a pathogenesis-based strategy for inhibiting degradation of the cartilage matrix. We investigated the hypothesis that controlled release of IL-1Ra using injectable, thermoreversible and complex coacervate combination gels as drug delivery systems might reduce matrix degradation in OA.**

***Methods*. Thermoreversible combination gels that can be injected into joints were formed in aqueous solution by making a complex coacervate with recombinant human IL-1Ra (anakinra) and cationic macromolecules, and this was followed by co-formulation with methylcellulose as a negative thermosensitive polysaccharide. Gels containing anakinra were positioned in the upper insert of a transwell system and human OA chondrocytes were placed in the lower compartment and then they were stimulated with IL-1β. The expression of matrix metalloproteinases (MMPs) was examined by performing real time PCR and ELISA.**

***Results*. Complex coacervation between anakinra and protamine was successfully completed. IL-1Ra was released from the gels in a sustained release pattern for extended periods with minimal initial bursts. IL-1β markedly enhanced the expression of MMP. The IL-1Ra released from the gels significantly inhibited the IL-1β-induced MMP expression in the chondrocytes.**

***Conclusion*. We developed and optimized a novel injectable and thermoreversible gel system for the controlled release of IL-1Ra, and this drug delivery system effectively inhibited the IL-1β-induced MMP expression of chondrocytes in a transwell system. Intra-articular local delivery of injectable and thermoreversible gels containing IL-1Ra into knees has the potential to provide prolonged therapy based on the pathophysiology of knee OA.**

***Key Words*. Drug delivery system, Interleukin-1, Interleukin-1 receptor antagonist, Methylcellulose gel, Osteoarthritis**

Introduction

Osteoarthritis (OA) is one of the most common causes of disability in the elderly (1), but the current treatment strategy is predominantly symptomatic (2). Recently, there has been a trend towards biological treatment based on the pathophysiology of OA in animal models (3-8) and even in human subjects (9).

IL-1 is a potent proinflammatory cytokine as well as the main cytokine causing articular cartilage destruction through the induction of matrix metalloproteinase (MMP) expression in chondrocytes in a paracrine and autocrine fashion (10). Furthermore, IL-1 decreases mRNA expression of alpha 1 (II) procollagen and suppresses the synthesis of Type II collagen and proteoglycan (11,12).

The biological effect of IL-1 is mediated by IL-1 receptors type I and decoy receptor type II, and balanced mainly by naturally occurring soluble IL-1 receptor antagonists (IL-1Ra)

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Correspondence : Yong-Hee Kim
Department of Bioengineering, College of Engineering, Hanyang University, 17 Haengdang-dong, Sungdong-gu, Seoul 133-791, Korea
E-mail : yongheekim@hanyang.ac.kr

(13,14). Recombinant human IL-1Ra anakinra (Kineret®; Amgen Inc., Thousand Oaks, CA, USA) was recently developed and approved for use in adults with rheumatoid arthritis (RA) in the US and Europe (15). Recombinant human IL-1Ra has proven effective for the treatment of experimentally induced OA (3). In a safety study in human OA, intra-articular injection (IAI) of anakinra was well-tolerated and showed some efficacy in terms of pain and function (9).

Recently, we developed thermoreversible combination gels that can be injected into joints and that are formed in aqueous solution by making a complex coacervate with anakinra and cationic macromolecules, followed by co-formulation with methylcellulose as a negative thermosensitive polysaccharide. Coacervation is a process during which a homogeneous solution of charged macromolecules undergoes a liquid-liquid phase separation, producing a polymer-rich, dense phase. Coacervation methods have been widely used for protein purification and drug delivery (16-18). Two oppositely charged macromolecules can form a complex coacervate by electrostatic interaction, resulting in a size increase. Complex coacervate formation of the target protein inside a gel increases both protein stability and gel viscosity and controls the release of protein from the gel. We described novel injectable thermoreversible combination gels based on a naturally occurring polysaccharide (high molecular weight methycellulose (HMw MC)), salting-out salt (ammonium sulfate (AS)) and complex coacervates. These systems showed promise for *in situ* gel depot protein drug delivery (19). In the present study, HMw MC was replaced with low molecular weight methylcellulose (LMw MC) for the purpose of increasing biocompatibility and elimination from the body. Due to its thermoreversible property, this system prepared in pure water was free-flowing at room temperature, but formed a gel depot at body temperature, making it a promising injectable therapy.

Two major components of articular cartilage, collagen and aggrecan, are the major targets of osteoarthritic changes, mainly by MMPs and aggrecanase (20,21). We investigated the effects of combination gels loaded with anakinra on the expression of major MMPs in OA (22), especially collagenase 1, 3 (MMP-1 and -3), gelatinase A and B (MMP-2 and -9), and stromelysin 1 (MMP-13) in human OA chondrocytes stimulated with IL-1β.

Materials and Methods

Materials

Human IL-1β was purchased from R&D Systems (Minneapolis, MN, USA), and human recombinant IL-1Ra (anakinra) was obtained from Amgen (Thousand Oaks, CA, USA). LMw MC (molecular weight=14,000, viscosity=15 cps), AS, and protamine (from salmon, grade V) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Human osteoarthritic chondrocyte

Articular cartilage samples for the preparation of chondrocytes were obtained from patients with knee OA undergoing total knee arthroplasty. The Hanyang University Institutional Review Board approved this study, and cartilage samples were obtained after informed consent was granted. Cartilage samples were cut into small pieces (about 2×2 mm), washed in DMEM media, and digested with a mixture of 1 mg/mL collagenase and 1mg/ml hyaluronidase solution for three hours. After filtering with mesh, the cell suspension was washed with DMEM twice and centrifuged at 250 g for five minutes. Cells were cultured and maintained with passage in DMEM supplemented with 10% FBS under regular culture conditions (37°C, 5% CO_2) until usage (third or fourth passage).

Thermoreversible gel preparation

Preparation of LMw MC aqueous solution. LMw MC powders were dissolved in half of the required volume of phosphate buffered saline (PBS) pre-heated at 95°C. After the remaining PBS was added, the solution was stirred for five minutes, stored on ice for five minutes, gently stirred for 30 minutes, and refrigerated.

Complex coacervate formation and turbidity measurements. Protamine was dissolved in PBS, pH 7.4. Anakinra was mixed with protamine solution at various weight ratios (protamine/anakinra (0.5, 1, 1.5, 2, and 4). The increase in turbidity of the mixture solutions was measured by absorbance at 450 nm (DU 730, Life science UV/Vis spectrophotometer, Beckman Coulter, Fullerton, CA, USA).

Preparation of thermo-reversible complex coacervate combination gels containing anakinra. Anakinra was loaded into the gel by mixing the complex coacervate PBS solution of protamine/anakinra with the 4% LMw MC/4.5% AS PBS solution.

Culture system

To investigate the effects of the combination gels loaded with anakinra on the biology of human OA chondrocytes, we used a Transwell® system (Corning Inc., Acton, MA, USA) with a 0.4 μm pore insert. To evaluate the long-term effects of gels loaded with anakinra, gels containing anakinra (10 μg or 100 μg) were positioned in the upper insert at day #0, and maintained in the culture room with daily changes of culture media to remove released anakinra. OA chondrocytes were

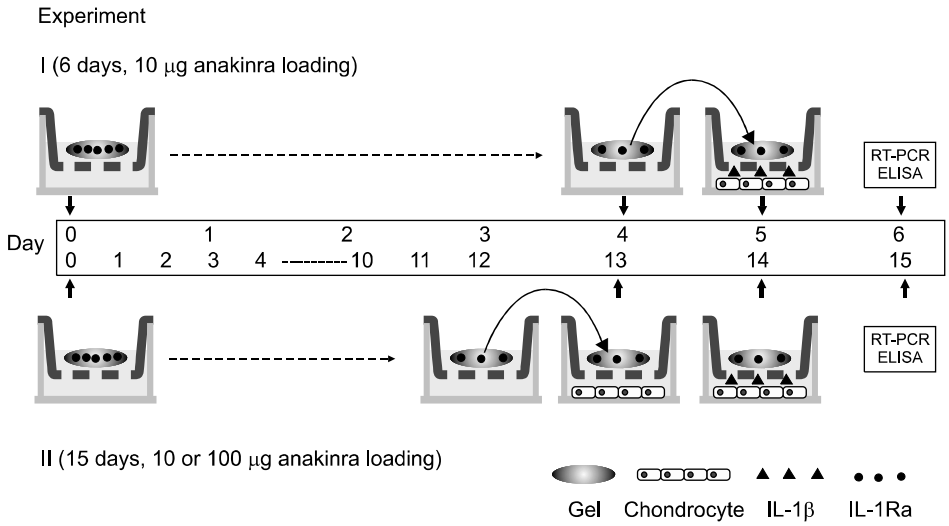

Figure 1. Experimental protocol. (1) Experiment I (6-days gel, 10 μg of anakinra, no coacervate): upper arm. Day #0: gel preparation and anakinra loading, Day #1～#6: daily change of media and measurement of the concentration of anakinra at the indicated days for the release pattern of anakinra, Day #4: transfer of gel into the upper compartment of the new system with plated chondrocytes on the lower compartment, and stimulation of chondrocytes with IL-1β (0.1 ng/ml) for 24 hours, Day #5: preparation of RNA and harvesting the supernatant for real time PCR and ELISA. (2) Experiment II (15-days gel, 10 or 100 μg of anakinra, coacervation with protamine): lower arm. Day #0: gel preparation and anakinra loading, Day #1～#15: daily change of media and measurement of the concentration of anakinra at the indicated days for the release pattern of anakinra, Day #13: transfer of gel into the new system with plated chondrocytes on the lower compartment, Day #14: stimulation of chondrocytes with IL-1β (0.1 ng/ml) for 24 hours, Day #15: preparation of RNA and harvesting the supernatant for real time PCR and ELISA. IL-1Ra: IL-1 receptor antagonist, RT-PCR: real time polymerase chain reaction, ELISA: Enzyme-Linked ImmunoSorbent Assay.

Table 1. Primer sets used for real time PCR

	Forward	Reverse
MMP-1	CATGCCATTGAGAAAGCCTTCC	AGAGTTGTCCCGATGATCTCC
MMP-2	ATAACCTGGATGCCGTCGT	TCACGCTCTTCAGACTTTGG
MMP-3	GCAGTTTGCTCAGCCTATCC	GAGTGTCGGAGTCCAGCTTC
MMP-9	GTCACCTATGACATCCTGCAGTG	CTTTCCTCCAGAACAGAATACCAGTT
MMP-13	GGACAAGTAGTTCCAAAGGCTACAA	CTTTTGCCGGTGTAGGTGTAGATAG
COL2A1	AAGCAGCTGGCAACCTCAAGAA	TGTTTCGTGCAGCCATCCTTCA
AGC	TTGCCAGCACCACCAATGTAAGTG	TGGTTCAGTAACACCCTCCACGAA
GAPDH	TCGACAGTCAGCCGCATCTTCTTT	GCCCAATACGACCAAATCCGTTGA

PCR: polymerase chain reaction, MMP: matrix metalloproteinase, COL2A1: type II collagen, AGC: aggrecan, GAPDH was used as the loading reference.

placed in the lower compartment at day #5 in Experiment I (without coacervation with protamine) or at day #13 in Experiment II (with coacervation). Chondrocytes were stimulated with IL-1β (0.1 ng/mL) simultaneously (Experiment I) or after 24 hours (Experiment II) of gel placement on the upper insert (Fig. 1). The culture supernatants and cells were collected for real time PCR and ELISA after 24 hours of stimulation with IL-1β. All experiments were conducted in at least three strains of chondrocytes in duplicate.

Total RNA extraction and real time polymerase chain reaction

Total RNA was isolated from cultured chondrocytes using RNAzolB (TelTest Inc., TX, USA) according to the manufacturer's instructions and quantified by spectrophotometer. The RNA was converted to cDNA using Reverse Transcriptase (Promega, WI, USA) and used as a template for real-time PCR. Real-time PCR was performed with a LightCycler (Bio-Rad Lab Inc., Hercules, CA, USA) using specific primers (Table 1) for GAPDH, type II collagen, aggrecan, MMP-1, -2, -3, -9, and -13.

Enzyme-linked immunosorbent assay

IL-1Ra released from the gel was measured by ELISA (Minneapolis, MN, USA). The levels of MMP-3 in culture supernatants were measured with a commercially available ELISA kit from R&D Systems (Minneapolis, MN, USA) according to the manufacturer's protocol.

Statistical analysis

Data were expressed as the mean±SD. Assays were performed in duplicate. Student's *t*-test was used for statistical evaluation, and p-values less than 0.05 were considered statistically significant.

Results

Coacervation between protamin and anakinra

Based on turbidity measurements of the protamine and anakinra mixture, the optimum conditions for efficient complex coacervate formation between protamine and anakinra included a weight ratio of 2 and an incubation time of three hours (Fig. 2).

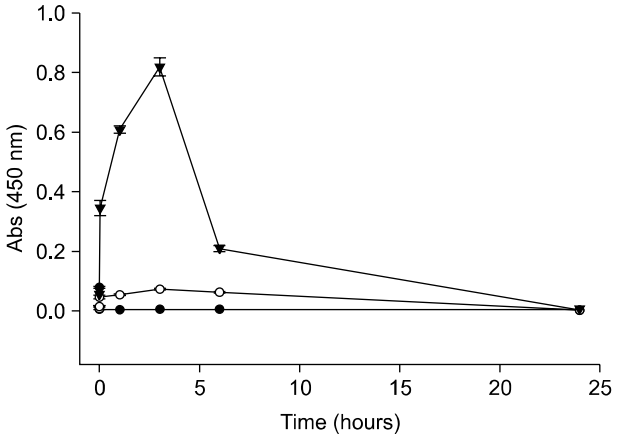

Figure 2. Turbidity curves of protamine and the IL-1Ra mixtures. Turbidity curve at various concentration in PBS, pH 7.4, 25°C (weight ratio of protamine to IL-1Ra: ●: 0.5, ○: 1, ▼: 2). The turbidity results indicated whether or not two macromolecules formed the coacervate. If the turbidity was increased, then coacervate was formed. At the weight ratio of 2, the turbidity was highly increased within 3 hours. Abs: absorbance.

Release of anakinra from thermoreversible complex coacervate combination gels

The release profile of anakinra from the gels showed a sustained release pattern through 15 days with minimal initial bursts with coacervation with protamine (Fig. 3). In Experiment I, however, without coacervation release pattern was steep. The concentration and cumulative percentage of released anakinra from the gels into culture supernatants are indicated in detail in Table 2 and Fig. 3.

Effect of the combination gels loaded with anakinra on gene expression of anabolic and catabolic genes

IL-1β (0.1 ng/mL) induced gene expression of MMPs. The combination gels alone, however, did not have a considerable

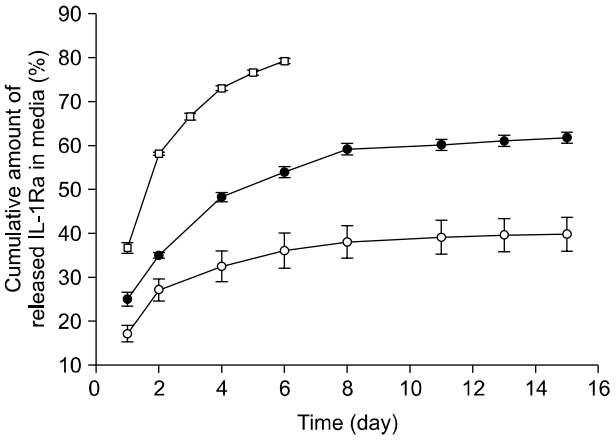

Figure 3. In vitro release of anakinra from the thermo-reversible, coacervate combination gel. The IL-1Ra released from the gel was measured by ELISA. Without coacervation with protamine, almost 80% of the loaded anakinra was released by Day #5, but coacervation made the release pattern slow down with a minimal initial burst. Only 60% and 40% of the loaded anakinra was released from the gels loaded with 10 μg and 100 μg of anakinra at Day #15, respectively. IL-1Ra: IL-1 receptor antagonist (□: 10 μg IL-1Ra, ●: 10 μg IL-1Ra+20 μg protamine, ○: 100 μg IL-1Ra+200 μg protamine).

Table 2. Concentration of the released anakinra from the gels into the culture supernatant

Experiment No.	Loading (μg)	Concentration of the released anakinra (ng/mL) at day #									
		#1	#2	#3	#4	#5	#6	#8	#11	#13	#15
I	10	3,661±120	2,144±82	848±34	640±53	361±11	258±6	–	–	–	–
II	10	2,496±152	1,006±164	–	1,313±102	–	571±48	520±10	100±8	91±5	67±5
	100	17,086±1,817	9,961±743	–	5,319±971	–	3,766±619	1,942±357	1,132±83	492±17	212±6

Values are the mean and standard deviation of the concentration of released anakinra into the culture media at the indicated day

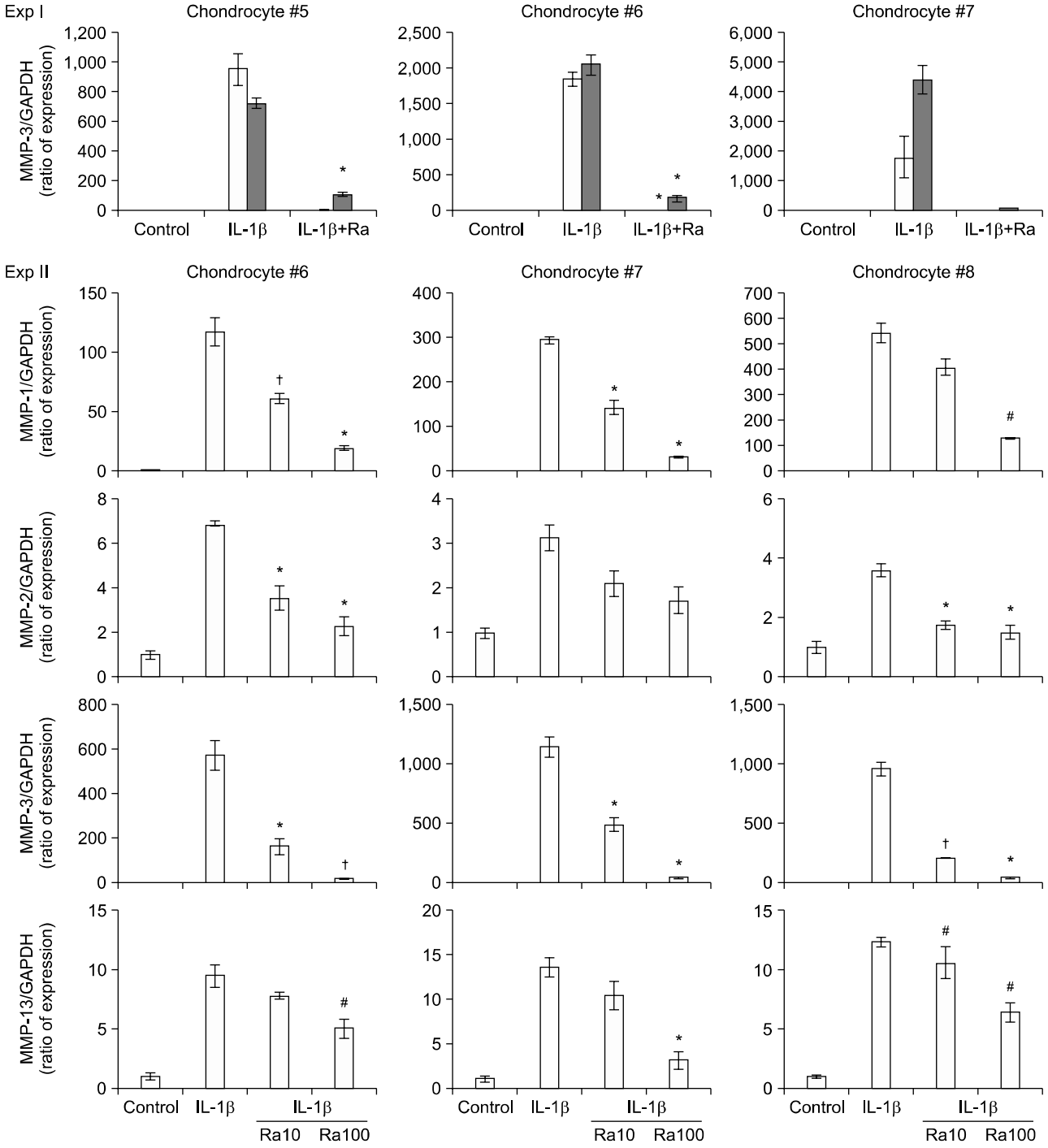

Figure 4. Inhibition of the gene expression of IL-1?-induced MMP-3 (Experiment I) and MMPs (Experiment II) by the anakinra released from the gel. Values are the mean and standard deviation of the indicated gene expression (duplicate measurement) relative to the untreated control. The asterisk indicates statistical significance (*$p<0.05$, $^{\dagger}p<0.001$ vs. the expression level induced by IL-1β, Student's t-test). $^{\#}$indicates the marginal statistical significance ($p=0.058$ for MMP-1, $p=0.059$, 0.057, 0.060 respectively for MMP-13). In Experiment I, we examined the effect of the combination gel itself on the gene expression. IL-1β + Ra indicate the stimulation with IL-1β and Ra300 without the gel in the blank bar, and the stimulation with IL-1β in the presence of the gel loaded with Ra100 in the filled bar. The gel itself (filled bar) did not influence on the result and the 6-days matured gels with anakinra suppressed the IL-1β-induced expression of MMP-3 (Experiment I). In Experiment II, the 15-days matured gels with anakinra significantly inhibited the IL-1β-induced expression of MMP-1, -2, -3 and -13 in a dose-dependent manner. MMP: matrix metalloproteinase, Ra10, 100 and 300: initial loading doses of IL-1 receptor antagonist (anakinra) at 10, 100, and 300 μg, respectively.

effect on gene expression (blank bar vs. filled bar in Fig. 4). Combination gels containing anakinra (10 μg) at Day #6 (Experiment I) dramatically inhibited IL-1β-induced MMP-3 expression (Fig. 4).

We decided to extend our experiments up to two weeks (Experiment II). In Experiment II, the stimulation of cultured chondrocytes with IL-1β for 24 hours was performed after 24 hours of gel replacement onto the upper insert to ensure sufficient anakinra release from the gels. The combination gels at Day #15 also considerably suppressed the IL-1β-induced expression of MMP-1, -2, -3, and -13 (Fig. 4), although all cell strains did not show statistically significant results compared to the expression level induced by IL-1β. MMP-9 expression was not significantly changed between stimulation with IL-1β and inhibition with anakinra (data not shown). COL2A1 expression was inhibited by IL-1β in two of three samples, but the decline was not significant and was not reversed with anakinra (data not shown). Aggrecan expression was down-regulated in all cases by IL-1β, and recovered significantly by anakinra in two cases (data not shown).

Effect of the combination gels on MMP-3 protein expression

IL-1β (0.1 ng/mL) induced the protein expression of MMP-3. In Experiment I (at Day #6) and II (at Day #15), MMP-3 protein expression induced by IL-1β was significantly inhibited with the combination gels with anakinra (Fig. 5). The combination gels alone had no considerable effect on protein expression.

Discussion

Based on the pathophysiology of OA, IL-1 has been considered a good target to modulate disease progression (23-25). Moreover, in an *in vitro* OA model and several studies using an animal model of OA, inhibition of IL-1 by gene transfer or IAI of IL-1Ra had a beneficial effect on both structural and histological modifications in OA (3-8). A pilot clinical study to investigate the safety of human IL-1Ra demonstrated that IAI of anakinra in patients with knee OA was well tolerated and improved pain and WOMAC scores (9). Unfortunately, anakinra has a very short terminal half-life of 4～6 hours after a single subcutaneous injection. Very recently, a multicenter, randomized, double-blind, placebo-controlled study of IAI of anakinra in OA of the knee was published (26). The authors reported that IAI of anakinra as a sin-

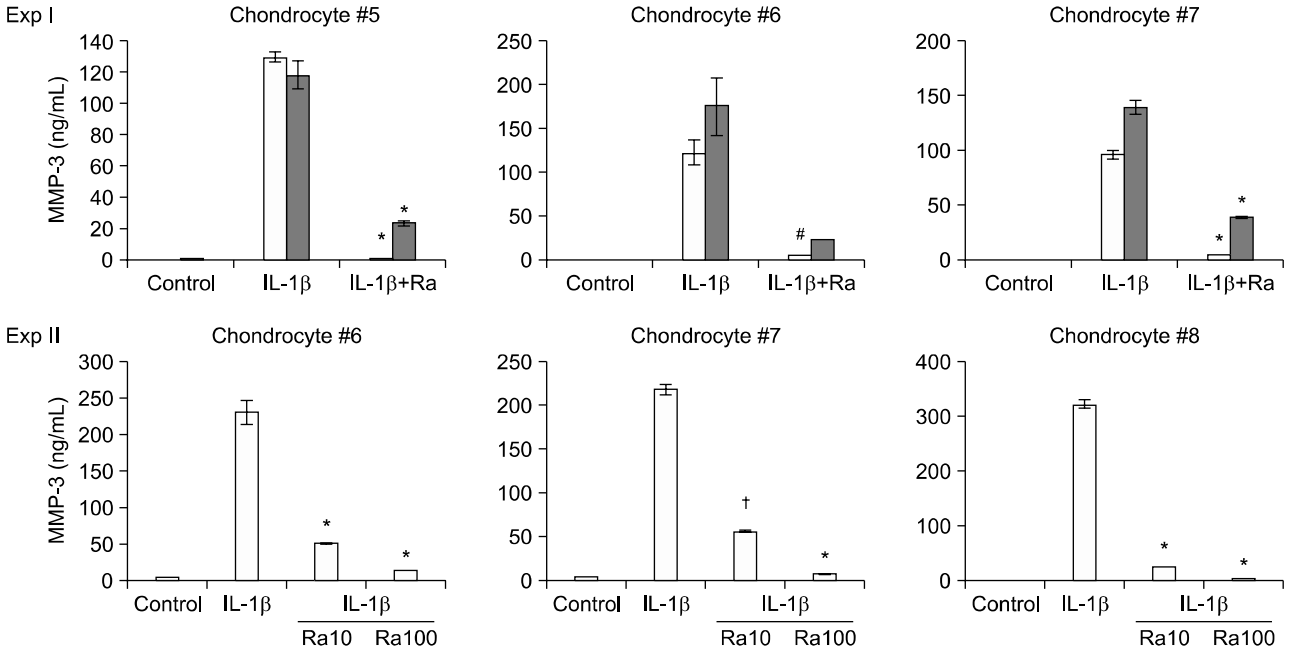

Figure 5. Inhibition of the protein expression of IL-1β-induced MMP-3 in the 6-days (Experiment I) or 15-days (Experiment II) matured gel with anakinra. The values are the mean and standard deviation of the measured concentration (duplicate) of MMP-3 (ng/ml). The asterisk indicates statistical significance (*$p<0.05$, $^{\dagger}p<0.001$ vs. the expression level induced by IL-1β, Student's t-test). $^{\#}$indicates marginal statistical significance ($p=0.055$ in Exp I). The gel itself (filled bar) did not have a considerable influence on the result (Experiment I). In Experiments I and II, the 6-days and 15-days matured gels with anakinra significantly inhibited the IL-1β (0.1 ng/ml)-induced expression of MMP-3 in a dose-dependent manner. The legend is same as that for Fig. 4.

gle dose of 50 mg or 150 mg into the knee joint was well tolerated, but did not improve OA symptoms compared with placebo. These results might appear disappointing with regard to the efficacy of biologics based on the pathogenesis of OA. Fifty-eight percent of patients, however, had Kellgren-Lawrence grade 3 OA, and the WOMAC pain score at day 4 was improved in the anakinra 150 mg arm. More importantly, anakinra was intra-articularly introduced only once, prompting the authors to suggest that IL-1 antagonists with longer action and more potency were needed to demonstrate the potential of IL-1 inhibition in the treatment of OA. Therefore, although significant improvement still existed three months after injection in the pilot clinical study (9), it may be unreasonable to expect a significant improvement in OA symptoms several months after a single IAI of anakinra into the knee.

Another issue in the application of biological agents is the route of administration. The IA route might be preferable in the treatment of OA due to the accessibility of injected IL-1Ra to superficial OA lesions of the cartilage, which is avascular, and the prevention of systemic and local adverse reactions caused by systemic administration (9).

Given the challenges described above, our system using a thermoreversible and complex coacervate combination gel for protein drug delivery, previously described in detail (19), is promising. In this study, we demonstrated the clinical feasibility of our in situ gel depot system in the treatment of knee OA. As shown Fig. 2 and Table 2, anakinra released more slowly from gels into culture media with coacervation with protamine. In Experiment II, the released concentration of anakinra (10 μg and 100 μg loaded) at Day #15 was about 33.5 and 106 ng/mL, respectively (because the released amount of IL-1Ra was a total of 67±5 and 212±6 ng/mL, respectively, during the last two days), which hypothetically corresponds to an excess of at least 300～1,000-fold over the amount of IL-1 ($<$1.0 to $<$100.0 pg/mL) (27,28) and an even greater excess of $>$30,000-fold over the amount of measured IL-1 (1.0 pg/ml) (29) in synovial fluid from patients with OA. Accordingly, our combination gels with anakinra (particularly 100 μg) might effectively inhibit IL-1 in the milieu of the joint cavity for a more prolonged time. If the gels could be applied repeatedly at specific intervals to cases of early OA with Kellgren-Lawrence scores $\leq$2, OA symptoms might be improved and the progression of cartilage destruction slowed. As expected, our gel system reduced the amount of anakinra to be injected and prolonged the effectiveness and half-life of anakinra. In fact, the amount of anakinra injected into the knee in a pilot study (9) and randomized, placebo-controlled trial (26) made the concentration of anakinra in the knee joint higher than necessary. The mean plasma concentration of anakinra several hours after administration, either subcutaneously or intra-articularly, was very similar (26,28). The effect of such an extremely high concentration of IL-1Ra on chondrocytes and synovial cells of the joint remains unknown. In contrast, our gel system maintains the necessary concentration over a certain period of time with a relatively small amount of anakinra. As shown in Fig. 3, the cumulative amount of anakinra released from the gels until Day #15 was less than 60% or 40% of the original amount of anakinra (10 μg or 100 μg, respectively) loaded into the gels. Changing the loading amount of anakinra or the ratio of protamine to anakinra could modulate the release pattern of anakinra from the gels.

Our study has some limitations. We only assessed the inhibitory effects of IL-1Ra on IL-1 β-induced gene and/or protein expression of MMPs. Recently, the role of aggrecanase in the degradation of cartilage has been illuminated (30), and the effectiveness of our combination gels with IL-1Ra on the expression of aggrecanase must be explored. We also need to evaluate the effect of locomotion on the properties of the gels, as well as biodegradation of the gels in the joint, since walking probably make the gels less functional. Since our gels are thermoreversible, they will form a stable gel in the joint cavity. Accordingly, specific gel placement, such as in the suprapatellar space of the knee joint and not in the joint space between the femur and tibia, could minimize the negative influence of locomotion on the properties of the gel. We plan to investigate the effectiveness of our injectable gel system in inhibiting the progression of cartilage destruction in an animal model of OA, shedding light on some of these unanswered questions. Finally, we have to say that this study has a limitation using cultured human chondrocytes, which undergo a change in their expression profile with increasing passage (31).

As mentioned recently (32), it is too early to say that anakinra is not effective for the treatment of OA. We should explore the alternative strategies for enhancing the activity of anakinra. These include the use of drug delivery system like our gel system to release anakinra very slowly, resulting in prolonged inhibition of IL-1 activity, and the trial of repeated injection of anakinra on a regular basis.

In the meantime, fully humanized monoclonal anti-IL-β antibody with a 28-day half-life was developed. This antibody will be contributable to the pathogenesis-based treatment strategy of OA, per se or with our drug delivery system, although at present approved only for cryopyrin-associated periodic syndromes (33,34).

Conclusion

We developed and optimized the injectable, thermoreversible, and complex coacervated combination gels containing human IL-1Ra anakinra, which inhibited IL-1β-induced expression of MMPs in human OA chondrocytes for an extended time period. This in vivo gel system may facilitate the clinical feasibility of an IL-1-based therapeutic strategy for OA.

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