Intra-articular Retention and Anti-arthritic Effects in Collagen-Induced Arthritis Model Mice by Injectable Small Interfering RNA Containing Hydrogel

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Small interfering RNAs (siRNAs) are expected to offer a means of treating rheumatoid arthritis (RA) because they allow the specific silencing of genes related to RA pathogenesis. In our previous study, we reported that the siRNA targeted against RelA (anti-RelA siRNA), an important nuclear factor-kappaB (NF-κB) subdomain, was an effective therapeutic in atopic dermatitis and RA model animals. In this study, to develop an intra-articular injectable gel formulation against RA, we prepared a hydrogel that contains anti-RelA siRNA, and determined the in vitro release profile (%) and in vivo intra-articular retention of fluorescence-labeled model siRNA, and the anti-arthritic effects of the anti-RelA siRelA containing hydrogel in RA model mice. We selected the silk protein, sericin (SC), as an aqueous gel base, as it is a biocompatible and useful for forming hydrogels without a cross-linker. We showed that fluorescence-labeled model siRNA was continuously released from SC hydrogel in vitro, and retained in the knee joint of rats after injection of siRNA hydrogel. In addition, the knee joint thickness, clinical severity and incidence (%) in collagen-induced arthritis (CIA) mice as RA model treated with anti-RelA siRNA containing hydrogel were more improved than untreated, anti-RelA siRNA solution and negative control siRNA containing hydrogel group. Therefore, the intra-articular injectable sericin hydrogel formulation containing of anti-RelA siRNA could be a great potential therapeutic in rheumatoid arthritis.

Key words rheumatoid arthritis; nuclear factor-kappaB (NF-κB); RelA; injectable gel formulation; hydrogel; small interfering RNA (siRNA)

Intra-articular administration of hyaluronic acid formulations is currently used to treat joint pain associated with rheumatoid arthritis (RA). A number of previous studies using animal models of joint inflammation have reported the efficacy of intravenous and subcutaneous administration in ameliorating joint inflammation. In addition, intra-articular administration has been reported to achieve high therapeutic effectiveness by improving the delivery and retention of drug compounds.1–2 Many recent studies have focused on the maintenance and sustained release of drugs from macromolecular hydrogels. We have previously succeeded in improving sustained release by integrating albumin and fibroblast growth factor-2 (FGF-2) using sericin (SC), a biodegradable and biocompatible silk protein, thereby forming a hydrogel without the use of a crosslinking reagent.3–5 Additionally, the sericin has biodegradability, biocompatibility, low-immunogenicity and non-toxicity.6,7 We also developed a hydrogel formulation including RelA-targeting small interfering RNA (siRNA) (siRelA). The siRelA retention was improved at the administration site after topical application in a mouse model of atopic dermatitis, and significant improvement of symptoms was observed upon application of the SC hydrogel in comparison with application of siRelA alone.8) This improvement in retention at the administration site may be attributed to the intrinsic moisture-retention and anti-inflammatory effects of the SC hydrogel. Nuclear factor-kappa B (NF-κB) plays an important RelA in gene expression control of the variety to participate in immune response, a stress reply, cell proliferation and differentiation and is a transcription inhibitor controlling cytokine agreement to participate in an allergic disease. In recent years, NF-κB decoy oligodeoxynucleotides (ODNs) have been reported to ameliorate RA symptoms in collagen-induced arthritis (CIA) mice and atopic dermatitis.8,9) Therefore, we focused that siRNA targeted against RelA, an important NF-κB subdomain and reported that the siRNA targeted against RelA (anti-RelA siRNA), an important NF-κB subdomain, was an effective therapeutic in atopic dermatitis and RA mouse model.10–12 In this study, to develop a RA-treatment injectable drug/siRNA retentionable formulation, an injectable SC hydrogel formulation containing siRNA was prepared and assessed in vitro sustained release and retention after intra-articular injection of a hydrogel formulation containing fluorescence-labeled model siRNA. Additionally, we also evaluated the therapeutic efficacy following intra-articular administration in a CIA mouse.

MATERIALS AND METHODS

Materials and Animals Sericin Hope SHC (SC purity is 98.5%, Kougensa Co., Ltd., Nagano, Japan) was used as the source of intact SC. The anti-RelA siRNA (siRelA; sense, 5'-GGUGCAAGAAGAGAGACAUUdTdT-3'), and negative control siRNA (siControl; sense, 5'-ACUCGCGCCGUAUGUACGUAdTdT3') were obtained from Cosmo Bio Co., Ltd. (Tokyo, Japan). The siControl did not affect any mRNA in humans, rats and mice. 10000MW Alexa Fluor®

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568-conjugated dextran (Alexa-dextran; AD) was obtained from Life Technologies (Carlsbad, CA, U.S.A.) as a fluorescence-labeled model siRNA. The Bovine type II collagen (CII) was purchased from Life Laboratory Company (Yamagata, Japan). The incomplete Freund's adjuvant (IFA) and complete Freund's adjuvant (CFA) were purchased from MP Biomedicals (CA, U.S.A.). Eight-week-old male Sprague-Dawley (SD) rats and DBA/1J mice were purchased from SLC (Shizuoka, Japan). All experiments with animals were carried out in accordance with a protocol approved by the Animal Care and Ethics Committee of the Tokyo University of Pharmacy and Life Sciences.

**Preparation of siRNA or AD Containing SC Hydrogel**
First, glass vials were filled with 50mL of distilled water, and water baths were heated until boiling. Then, 1g of SC was added to the heated distilled water and dispersed by shaking for 20min. We previously studied the molecular weight of SC after heating under above conditions using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and there are three kinds of molecular weight of intact SC proteins (180, 250, 400kDa), which is able to form hydrogel with water in the soluble components. Next, insoluble substances were separated by centrifugation, and the soluble components were collected and used as 2% SC solution. Then, this solution was combined with either AD or siRNA solution, and stored overnight at 4°C to produce a 1% SC hydrogel containing either AD or siRNA. The SC hydrogel was cooled to room temperature for use at the time of evaluation.

**In Vitro Release Profile of AD from SC Hydrogel**
The 1% SC/AD gel was added to a 6-well plate and then heated to 37°C. Then, 5mL of phosphate-buffered saline (PBS) was added to the wells. The plates were then sealed with parafilm and stored in darkness. The temperature was maintained at 37°C. Subsequently, 400µL of PBS was collected, and an additional 400µL of PBS was added after each fixed period while maintaining the temperature at a constant value. Insoluble SC was separated from the collected PBS via centrifugation, and then, 200µL of PBS was added to each well of a 96-well plate. Fluorescence (excitation: 578 nm, emission: 603 nm) was then measured using a microplate reader. The cumulative AD released rate for each period was calculated based on the fluorescence of each sample.

**In Vivo Retention of AD Containing SC Hydrogel in Knee Joint**
Following anesthesia, 100µL of AD solution and SC/AD gel were injected into knee joints of hind legs of rats using a 26G-needle syringe. We confirmed that the 1% SC/AD gel can pass though the syringe of 26G size on keeping gel state. Following the injection and after a fixed period, the hind legs were removed, and the fluorescence intensity at the injection site in the knee joint was examined using a fluorescence imaging apparatus (Maestro™, Kurabo Industries, Ltd., Osaka, Japan). In addition, fluorescence intensity was calculated using photographed fluorescent images of the same areas in each sample.

**Therapeutic Effects in CIA Mice**
Eight-week-old male DBA/1J mice were anesthetized. Bovine Collagen II (CII) is used as the antigen, the CFA is Complete Freund's Adjuvant (M.Tuberculosis, WA, U.S.A.) and IFA is Incomplete Freund's Adjuvant (MP Biomedicals, CA, U.S.A.). An equal volume of CFA or IFA and CII solution (2mg/mL) were provided and homogenized (13000rpm, 3min) while dropping CII to the CFA or IFA under ice-cooling to prepare an emulsion. The 50µL of an emulsion of CII and CFA was administered intradermally at the base of the tail using a 1mL syringe with a 23G needle, resulting in sensitization. Subsequently, after 21d from sensitization (day 0), 50µL of an emulsion of CII and IFA was administered intradermally at the base of the tail as second immunization, and CIA mice were generated by administering immunity boosters.

The CIA mice were divided into five groups ($n=5$): an untreated group receiving RNase-free water, a group receiving SC hydrogel alone, a group receiving SC hydrogel containing siControl (siControl-SC hydrogel group), a siRelA solution group, and a siRelA-SC hydrogel group. Each group was received 30µL of injection of each treatment into the knee joints of both hind legs via a syringe with a 26G needle while under anesthesia on days 0, 2, 5, 7, 9, and 12 (per injection dosage administered to each knee joint: siRelA: 5µg/30µL).

The CIA mice were evaluated by assessing the clinical score, degree of swelling, and gross examination of the hind knee joints, as well as by examining tissue samples. Clinical score was calculated by scoring the right and left hind legs of each mouse on a scale of 0–6, and a mean score of 12 points was considered for each group for each test period. Specifically, the rate of increase in leg swelling was scored as follows: rates up to 105%: score of 0, rates of 105–125%: score of 1, rates of 125–150%: score of 2, and rates of 150% or higher: score of 3. Furthermore, toe inflammation (redness and swelling) was assessed on a scale of 0–3. The hind legs of each mouse were assessed, and the 12-point score total was used as the clinical score. In addition, mice with clinical scores of 4 or higher (maximum 12) were established as CIA-incidence mice, and the onset rate for each test period was calculated for each group. The thickness of the hind legs was measured using a thickness gauge. After initiation of sensitization, measurements were started on day $-7$, and were taken three times weekly after the administration of immunity boosters on day 0. Thickness on day 21 was established as the baseline, and the rate of increase in thickness was calculated. Further, after gross examination of the hind paw on day 14, the photographs of hind legs were taken and observed.

**Statistical Analysis**
The results of the experiments are represented as means±standard errors (S.E.). Pair-wise comparisons between treatments were made using Student's $t$-test. Comparisons between multiple treatments were made using ANOVA, followed by Dunnett's test. Group differences were considered to be statistically significant when $p<0.05$.

**RESULTS AND DISCUSSION**

**In Vitro Release Profile of Alexa-Dextran from SC Hydrogel**
Figure 1 shows the release profile of Alexa-dextran of 10000 molecular weight from SC gel over time. The SC hydrogel continuously released Alexa-dextran for 48h after the start of the study and then around 50%, suggesting that Alexa-dextran can be continuously released to the injection tissue by formed SC hydrogel formulation.

**In Vivo Retention of SC Hydrogel in Knee Joints**
There are some differences between in vitro and in vivo situation on release profile. In general, the degradation of intact SC is caused by hydrolysis of its hydrophilic region, and immune-related cells, such as macrophages and peptidase are known.
causes of hydrolysis in previous report. In addition, we previous reported that the SC hydrogels was decreased over time in the skin of rats.

Therefore, we next assessed the retention of the SC/AD gel following intra-articular administration to SD rat using in vivo imaging system. The rat is suitable size to observe the detail about intra-articular retention. Figure 2A shows the fluorescent images from day 0 and after day 14, and Fig. 2B shows the luminosity calculated based on the fluorescent images in Fig. 2A.

In the groups receiving solutions, AD fluorescence was more widespread around the injection site immediately after administration, while in the groups receiving SC hydrogels, fluorescence was strongest in a narrow region (Fig. 2A). No efflux was observed in the groups receiving the solutions when the knee joint tissue was removed after administration, and AD appeared to adhere to the surrounding tissue. Similarly, AD appeared to adhere to the surrounding tissue in the groups receiving SC hydrogels, but degradation of the SC/AD gel, which contained less water, was observed. Furthermore, a significant decrease in fluorescence was noted after day 7 in the groups receiving the solutions. By contrast, although a chronological decrease in fluorescence was noted in the SC hydrogel administration group, intra-articular retention of AD was noted on day 14 in the SC group. Fluorescence intensity on days 7 and 14 was significantly higher in the SC groups compared to the solution group.

Fig. 1. In Vitro Release Profile of Alexa-Dextran from SC Hydrogel

SC hydrogel containing Alexa-dextran (Mw: 10000) were added into PBS. PBS that contains Alexa-dextran released from SC hydrogel were collected after 0.25, 0.5, 0.75, 1, 3, 6, 10, 24, 48h from the beginning. Each point represents the mean±S.D. (n=3).

Fig. 2. Intra-articular Retention of Alexa-Dextran Containing SC Hydrogel in Knee Joint

SD rats were intra-articular injected of Alexa-dextran solution or Alexa-dextran containing SC hydrogel. (A) Observation of the fluorescence in the knee joints by in vivo fluorescence imaging system. (B) Mean brightness of images of Fig. 2A. Each bar represents mean±S.E. (n=3). **p>0.05, *p<0.05, **p<0.01.

Fig. 3. Clinical Score and Incidence (%) of CIA Mice after Treatments by siRelA Containing SC Hydrogel

(A) Clinical score was calculated 0–6 points for each hind paw, totally 12 points. Leg swelling: rates up to 105%; score of 0, rates of 105–125%; score of 1, rates of 125–150%; score of 2, and rates of 150% or higher: score of 3. Toe inflammation (redness and swelling): each score 0–3. These involve thickness of ankle joints and swelling of hind finger as subjects. (B) Incidence (%) of RA in CIA mice. Above 4 points of clinical score, it deemed to incidence the RA. Each point represents mean±S.E. (n=5). **p<0.01 versus untreated.
than in the solution groups. Together, these findings indicate that the SC hydrogel may be used in formulations for intra-articular administration owing to its superior retentiveness.

**Therapeutic Effects of siRelA-Containing SC Hydrogel in CIA Mice** Figure 3 shows the rate of CIA incidence and the shifts in clinical score by each treatment group. As a result, the untreated group developed CIA faster than did the other groups, and the onset rate was 100% by day 11 for both the untreated group and the siRelA solution group (Fig. 3A). The reason for this result was suggested that free siRNA couldn’t suppress the RelA mRNA because it was not stable against the fluid of immune-activated knee joints. In contrast, the siControl/SC hydrogel group, and the siRelA/SC hydrogel group exhibited lower onset rates. The siRelA/SC hydrogel group in particular exhibited an onset rate of 40% in comparison with the onset rates of 60% and higher in the other groups, demonstrating a clear reduction in the onset rate. As shown in Fig. 3B, the significant declines in clinical scores were observed after day 7. Figure 4 shows the increase of thickening degree at the leg joints. As a result, the siRelA/SC hydrogel group demonstrated significant reduction in joint-site thickening after day 7 in comparison with the untreated group. Figure 5 shows the gross images of mouse hind legs. The untreated group and the siRelA solution group exhibited marked joint thickening, swelling, and reddening of the toes. In contrast, in the siControl/SC hydrogel group, joint thickening and toe swelling declined. Furthermore, almost no symptoms such as thickening or swelling were observed in the siRelA/SC hydrogel group, indicating a significant suppressive effect against RA.

In general, naked siRNA is known to be difficult to demonstrate the therapeutic effects in vivo model. However, in our previous study, the naked siRNA showed the intracellular uptake ability (5–10%) and the suppression of RelA mRNA level (30–40%) in LPS-stimulated immune-related cells, which are increased in immune-activated knee joint. In addition, it was reported that most of SC hydrogel was remained in rat skin on 3 d after injection. In this study, since the treating siRelA/SC gel was injected once two or three days, siRelA concentration and stability in topical knee joint could become higher than siRelA solution because of the protection by SC hydrogel in this treatment schedule. Furthermore, it was reported that sericin has anti-inflammation effects itself. Therefore, these results show that, although intra-articular administration of siRelA as a solution was unable to improve the symptoms sufficiently, combining it with the SC hydrogel improved retention, siRelA uptake and suppression.
of the RelA mRNA expression in immune-related cells, and consequently, led to higher efficacy. We would like to consider further studies are needed in order to indicate more detail the mechanism of SC hydrogel or actually knockdown effect or retention of siRNA in further research.

CONCLUSION

The SC hydrogel formulation demonstrated injectable by the 26G needle-syringe and high retention in injection site when administered locally to the joints. Moreover, this SC hydrogel incorporated anti-NF-κB (RelA) siRNA, leading to a marked anti-arthritis effects in a mouse model of arthritis. Therefore, it may be useful and safety system as an intra-articularAndreable formulation for RA treatment. SC-based hydrogels are essential for enhancing the efficacy of locally administered biopharmaceuticals such as nucleic acids, and can serve as a biomaterial foundation for the development of new treatments for joint-related conditions.

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Conflict of Interest The authors declare no conflict of interest.

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