Arthritic Joint-Targeting Small Interfering RNA-Encapsulated Liposome: Implication for Treatment Strategy for Rheumatoid Arthritis

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ABSTRACT

RNA interference, mediated by small interfering RNA (siRNA), is effective in silencing genes with a high degree of specificity. To explore the therapeutic potential of systemically administered siRNA for rheumatoid arthritis (RA), we tested the complex of siRNA and the recently developed wrapsome (siRNA/WS) containing siRNA-encapsulated liposome in mice with collagen-induced arthritis (CIA). Mice with CIA received an intravenous injection of Cy5-labeled siRNA/WS. Fluorescence stereoscopic microscopy and flow cytometry were used to assess the siRNA/WS tissue distribution. The efficacy of siRNA-targeting tumor necrosis factor (TNF)-α/WS in CIA was evaluated by arthritis score. TNF-α mRNA levels in the joints were measured by real-time reverse transcriptase-polymerase chain reaction. The intensity of Cy5 fluorescence was higher in arthritic joints than in nonarthritic sites in Cy5-siRNA/WS-treated mice and remained higher up to 48 h after injection, compared with that in naked Cy5-siRNA-treated mice. Cy5 fluorescence intensity was higher in synovial cells than in splenocytes, bone marrow cells, and peripheral blood leukocytes. The majority of Cy5-positive synovial cells were CD11b+ with only a few CD3+ cells. Treatment with TNF-α siRNA/WS resulted in significant decreases in severity of arthritis and TNF-α mRNA level in the joints compared with control siRNA/WS. In conclusion, the use of our WS allowed efficient and targeted delivery of siRNAs to arthritic joints. The siRNA/WS was mainly incorporated into CD11b+ cells, including macrophages and neutrophils, in the inflamed synovium, suggesting its potential therapeutic effects in RA by silencing the expression of inflammatory molecules produced by these cells.

Introduction

RNA interference, mediated by small interfering RNA (siRNA), is a powerful way to silence genes with a high degree of specificity and is a potential therapeutic approach in various diseases including viral infections and proliferative vascular retinopathies (de Fougerolles et al., 2007; Whitehead et al., 2009). To apply this technology to the treatment of rheumatoid arthritis (RA), it is important to develop means to deliver siRNA via systemic injection to multiple affected joints. The delivery tool should be stable and protect siRNA from nuclease attack in vivo. Our group used the wrapsome (WS), which was recently designed with a core composed of a cationic lipid bilayer and siRNA complex enveloped in a neutral lipid bilayer with polyethylene glycol on the surface, as a vehicle for siRNA delivery (Yagi et al., 2009). In contrast to the widely used cationic liposomes, the WS is unique in that it is uncharged. The cationic liposomes are prone to bind in vivo to plasma proteins because of their cationic nature. Because the WS carries a neutral surface charge, the loss of WS function by the plasma protein is less likely. For the same reason, attachment of the WS to the endothelial cell membrane and its entrapment by the reticuloendothelial systems are avoidable, which can otherwise lead to adverse effects, including embolism, a complication reported with the use of cationic liposomes (Litzinger et al., 1996). In addition, the surface pegylation of the WS confers the long half-life of the WS in the systemic circulation (t1/2 of 17.6 h). Investigators in our group have recently demonstrated that the use of the WS results in accumulation of

ABBREVIATIONS: siRNA, small interfering RNA; RA, rheumatoid arthritis; WS, wrapsome; TNF-α, tumor necrosis factor-α; CIA, collagen-induced arthritis; mAb, monoclonal antibody; RT, reverse transcriptase; PCR, polymerase chain reaction; MFI, mean fluorescence intensity; IL, interleukin.
siRNA in tumors in vivo without any signs of significant toxicity (Yagi et al., 2009).

In the present study, we evaluated the accumulation of siRNA/WS complex into the inflamed joints of mice with experimentally induced arthritis. In addition, the therapeutic potential of the siRNA/WS complex targeting tumor necrosis factor (TNF)-α, which is produced primarily by macrophages in the synovia of the affected joints, was assessed in the arthritic mice.

Materials and Methods

Preparation of siRNA-Encapsulated Liposome. All siRNAs including fluorescence-labeled randomized siRNA were synthesized and purified by Hokkaido System Science (Hokkaido, Japan). The siRNA sequences were as follows: control randomized siRNA sense 5′-GGU ACA CAU GUG CAC ACA CdTdT-3′ and antisense 5′-GUG UGU GCA CAU GUG UAC CdTdT-3′; and TNF-α-siRNA sense 5′-GUG CCU AUG UCU CAG CCU CdTdT-3′ and antisense 5′-GAG GCU AGA UAG GCA CdTdT-3′ (Schiffelers et al., 2005). Preparation of the complex of siRNAs and WS, which was developed as delivery system for siRNAs, was described previously (Yagi et al., 2009). The siRNA/WS complex consists of siRNA and cationic lipids enclosed by a neutral lipid bilayer containing egg phosphatidyl choline and is ~100 nm in diameter.

Imaging of siRNA/WS Accumulation in Arthritic Joints of Mice with Collagen-Induced Arthritis. Murine collagen-induced arthritis (CIA) was induced in 8-week-old male DBA/1J mice (Charles River Japan, Tokyo, Japan) by immunization twice with bovine type II collagen (Collagen Research Center, Tokyo, Japan) and complete Freund’s adjuvant (Difco, Detroit, MI) (Nanki et al., 2005). From the day of the second immunization (day 0), mice were examined for signs of joint inflammation. The severity of arthritis was evaluated using the following clinical scoring method (Nanki et al., 2005): 0, normal; 1, erythema and mild swelling confined to the midfoot or ankle joint; 2, erythema and mild swelling extending from the ankle to the midfoot; 3, erythema and moderate swelling extending from the ankle to the metatarsal joint; and 4, erythema and severe swelling of the ankle, foot, and digits. The clinical score was defined as the sum of the scores of all four paws of each mouse. At day 7, CIA mice showing joint inflammation scored more than 2 with the above definition at least in a paw, were randomly injected either with a single injection of Cy5-labeled siRNA/WS, naked Cy5-labeled siRNA, or saline via the tail vein. Considering the limited sensitivity for detection of fluorescence in the in vivo setting, the doses of the Cy5-labeled siRNA were selected to be 50 and 150 μg for whole-body imaging (Yagi et al., 2009). Cy5 fluorescence was monitored for up to 96 h after administration using a fluorescence stereoscopic microscope (Macro Imaging System, Nippon Roper, Tokyo). The level of fluorescence-labeled siRNA in the affected joints was quantified by analyzing regions of interest using image analysis software (Nippon Roper). Approval for the experimental protocol was obtained from the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

Analysis of siRNA/WS Tissue Distribution in the CIA Mice. At day 7, CIA mice showing joint inflammation as described above were randomly injected either with 50 μg of Cy5-labeled siRNA/WS, naked Cy5-labeled siRNA, or saline via the tail vein. On the basis of the results of the whole-body imaging, the dosage of Cy5-labeled siRNA/WS for fluorescence-activated cell sorting analysis was selected. Twenty-four hours later, tissues (synovium, spleen, and bone marrow) were harvested, and single cell suspensions were prepared using standard procedures. Synovial tissues were harvested from arthritic joints (scored more than 2). Peripheral blood leukocytes were isolated by depletion of red blood cells with NH4Cl hemolytic solution. The cells were analyzed for Cy5 fluorescence using a FACScan (BD Biosciences, San Jose, CA). The following mAbs were used for staining: CD11b-phycocerythrin (eBioscience, San Diego, CA), F4/80-fluorescein isothiocyanate (eBioscience), CD3-fluorescein isothiocyanate (BD Pharmingen, San Diego, CA), and CD19-phycocerythrin (eBioscience).

Analysis of the Effect of TNF-α siRNA/WS in CIA Mice. At day 7, mice were divided into three groups: control (PBS), TNF-α-siRNA, and control. The levels of TNF-α mRNA were measured by real-time RT-PCR assay using TaqMan probes (TNF-α (Mm00443258) and glyceraldehyde-3-phosphate dehydrogenase (Mm99999915) (Applied Biosystems, Foster City, CA). Amplification was performed in a thermal cycler (ABI Prism 7000; Applied Biosystems).

Statistical Analysis. Data were expressed as mean ± S.E.M. For comparing clinical scores between groups, the nonpaired Student’s t test was used. For comparing incidence of arthritis between groups, Fisher’s exact test was used. Analyses were performed using StatView (Abacus Concepts, Berkeley, CA). P < 0.05 was considered significant.

Results

Accumulation of Systemically Administered siRNA/WS in Arthritic Joints of Murine CIA. The potential of the WS as a delivery tool of siRNA was investigated in CIA. At day 7, when the CIA mice developed arthritis (scored more than 2 at least in a paw), they were given injections with either fluorescence (Cy5)-labeled siRNA/WS or naked Cy5-labeled siRNA (50 or 150 μg). After 12 h, observation under the stereoscopic microscope showed accumulation of fluorescence in the arthritic joints but not in nonarthritic sites in mice treated with Cy5-siRNA/WS (150 μg), and the levels of the accumulation appear higher in severely swollen joints compared with that in less swollen or normal joints (Figs. 1, A and B). Although the intensity was weaker, fluorescence was detected in arthritic joints in mice treated with 50 μg of Cy5-siRNA/WS (data not shown). In mice treated with naked Cy5-siRNA (150 μg), although a low level of fluorescence was observed in arthritic joints immediately after the injection, the fluorescence disappeared within 12 h (Fig. 1C). The intensity of the fluorescence level remained high up to 48 h after injection in the arthritic joints of the mice treated with Cy5-siRNA/WS (Fig. 1D). Although the intensity was weaker, fluorescence was detected in arthritic joints in mice treated with 50 μg of Cy5-siRNA/WS (data not shown). In contrast, the intensity of the fluorescence level in the arthritic joints of the mice treated with naked Cy5-siRNA (150 μg) was much lower than that in the mice treated with Cy5-siRNA/WS (Fig. 1D).

Next, the tissue distribution of systemically administered Cy5-siRNA/WS was examined in the CIA mice. At 24 h after injection of 50 μg of Cy5-siRNA/WS, naked Cy5-siRNA, or saline, the Cy5 fluorescence levels in cells from the synovium, spleen, bone marrow, and peripheral blood leukocytes were examined by flow cytometry. Compared with that in saline-injected mice, the fluorescence intensity of Cy5 was higher in cells harvested from the
synovium of naked Cy5-siRNA-injected CIA mice (Fig. 2A).
Moreover, the Cy5 intensity was significantly enhanced in
synovial cells from mice injected with Cy5-siRNA/WS compared with that in those injected with naked Cy5-siRNA, with the ratio of mean fluorescence intensity (MFI) (Cy5-siRNA/WS versus naked Cy5-siRNA) of 4.4 (Fig. 2B). The Cy5 intensity in bone marrow cells and peripheral blood leukocytes of naked Cy5-siRNA-injected CIA mice was also higher; however, the Cy5 intensity in mice injected with Cy5-siRNA/WS was not enhanced (MFI ratios 0.97 and 0.57, respectively). Of note, the MFI of Cy5 was much higher in synovial cells from Cy5-siRNA/WS-injected mice compared with bone marrow cells and peripheral blood leukocytes (Fig. 2B). On the other hand, the fluorescence intensity was not significantly altered in splenocytes.

Next, we determined the cells that incorporate the Cy5-siRNA/WS in the synovium. At 24 h after injection, the synovial cells of mice treated with naked Cy5-siRNA or Cy5-siRNA/WS were analyzed by flow cytometry after staining with anti-CD11b, -F4/80, -CD3 or -CD19 mAbs. In the Cy5-siRNA/WS-injected mice, 81% of the synovial cells were Cy5-positive, and most of the Cy5-positive cells (>75%) were

![Fig. 1. Accumulation of systemically administered siRNA/WS in arthritic sites. At day 7 after induction of CIA, mice with arthritis (scored more than 2 at least in a paw) were given injections of saline, naked Cy5-siRNA, or Cy5-siRNA/WS. A, mice were examined 12 h later for accumulation of the Cy5-fluorescence with a stereoscopic microscope. The arrows indicate the sites where the fluorescence was observed. B, arabic numerals on each paw indicate arthritis score (top), and arrows show the accumulation of the fluorescence observed in the corresponding paws (bottom). C, fluorescence observed in arthritic hind paws of mice injected either with naked Cy5-siRNA (150 μg/bODY) or Cy5-siRNA/WS (150 μg/bODY) at 0 min (immediately after the injection) and 12 h. D, time course of the fluorescence intensity in arthritic hind paws (arthritic score = 3) of mice injected with naked Cy5-siRNA (150 μg/bODY) or Cy5-siRNA/WS (150 μg/bODY). Data are the mean ± S.E.M.; n = 3. A–C, representative photographs of eight mice are shown.]

![Fig. 2. Tissue distributions of systemically administered siRNA/WS. At day 7 after induction of CIA, mice with arthritis (scored more than 2 at least in a paw) were given injections saline, naked Cy5-siRNA, or Cy5-siRNA/WS. A, twelve hours after the injection, the levels of Cy5 fluorescence in cells extracted from the synovium, spleen, bone marrow, and peripheral blood leukocytes were analyzed by flow cytometry after staining with anti-CD11b, -F4/80, -CD3 or -CD19 mAbs. In the Cy5-siRNA/WS-injected mice, 81% of the synovial cells were Cy5-positive, and most of the Cy5-positive cells (>75%) were

![naked Cy5-siRNA-treated mice](#) Cy5-siRNA/WS-treated mice

![Cy5 positive cells](#) CD11b+ F4/80+ CD3+
CD11b+. In addition, 92% of CD11b+ and 67% of F4/80+ cells were Cy5-positive, whereas, only 20% of CD3+ cells were Cy5-positive. Although the majority of CD11b+ cells in the synovium of naked Cy5-siRNA-treated mice were also Cy5-positive (82%), fewer F4/80+ and CD3+ cells were Cy5-positive (1 and 10%, respectively) compared with Cy5-siRNA/WS-treated mice (Fig. 2C). CD11b+ synovial cells in CIA mainly consisted of macrophages (70%) and secondary dominant neutrophils (30%) (data not shown). Of the synovial cells, very few were CD19+ (less than 0.1%).

These results suggest that both selective delivery and retention of Cy5-siRNA to inflamed synovium could be achieved using the WS as a carrier of the siRNA and that systemically administered siRNA/WS would efficiently target macrophages and neutrophils in inflamed joints.

**Efficacy of siRNA Targeting TNF-α/WS for CIA.** Experimental evidence indicates that functional blockade of TNF-α effectively inhibits CIA (Kim et al., 2002). Thus, we analyzed the effect of systemic administration of siRNA/WS targeting TNF-α on CIA. Mice treated with TNF-α siRNA/WS at 1 and 10 μg showed substantially decreased and delayed incidence of arthritis over those treated with control siRNA/WS (P < 0.05, TNF-α siRNA/WS 10 μg versus control 10 μg, at day 7) (Fig. 3A). Treatment with 10 μg of siRNA/WS targeting TNF-α significantly lowered the disease severity expressed as clinical arthritis score and paw thickness, compared with control siRNA/WS (Fig. 3, B and C). On the other hand, naked TNF-α siRNA had no effect on CIA (data not shown). In addition, TNF-α mRNA levels in the paws of TNF-α siRNA/WS (10 μg)-treated mice were significantly lower than those in mice treated with control siRNA/WS (Fig. 3D). No signs of toxicity were observed in either control siRNA/WS or TNF-α siRNA/WS-treated mice. These results suggest that down-regulation of TNF-α expression in the synovial tissue by systemically administered TNF-α-targeting siRNA/WS inhibited CIA.

**Discussion**

In this study, we showed that systemically administered siRNA/WS complex accumulated in arthritic joints in murine CIA, where the complex was preferably incorporated into CD11b+ macrophages and neutrophils. Moreover, treatment with siRNA/WS targeting TNF-α ameliorated CIA.

RA is characterized by chronic synovitis affecting multiple joints, and synovial macrophages play central roles in the pathogenesis by producing various proinflammatory cytokines such as TNF-α, interleukin (IL)-1, and IL-6. Treatment with anti-TNF-α mAb was effective for murine CIA (Williams et al., 1992). Moreover, TNF-α blockade was efficacious in the treatment of RA (Smolen et al., 2007). However, systemic administration of TNF-α inhibitor could impair the immune system, which increases the development of infectious diseases (Komano et al., 2011), including reactivation of latent infections such as tuberculosis (Keane et al., 2001). Therefore, selective delivery of siRNA that targets macrophages in the affected synovia at multiple anatomical sites is an ideal strategy. Our results demonstrated that this goal can be achieved using the siRNA/WS formation, and such treatment might be potentially useful for RA in humans by inhibition of cytokine production by synovial macrophages. Our method could also target other proinflammatory cytokines or intracellular molecules in the synovial macrophages that are known to play some roles in chronic joint inflammation (Mosser and Edwards, 2008).

In arthritic synovium, incorporation of siRNA/WS was observed in a majority of the CD11b+ cells and in more than half of the F4/80+ cells, but only in a few in the CD3+ cells. CD11b+ synovial cells in our CIA model mainly consisted of macrophages and neutrophils. It was reported that F4/80+ synovial cells were mature macrophages (Morris et al., 1991). The mechanism of the selective incorporation of siRNA/WS in macrophages and neutrophils in the inflamed synovium is not fully understood. It is unlikely that the selective siRNA delivery by the siRNA/WS complex depends simply on the innate phagocytic capability of the cells. Investigators in our group reported that the complex was able to transduce therapeutic siRNA selectively into cancer cells in mice in vivo (Yagi et al., 2009). It is possible that the siRNA/WS is preferentially delivered and enters the affected synovium as a result of increased angiogenesis and vascular permeability in such inflammatory tissues. Indeed, the size of the siRNA/WS

![Image](https://i.imgur.com/5Q5Q5Q5.png)

**Fig. 3.** Effects of systemic administration of siRNA targeting TNF-α/WS on CIA. A–C, TNF-α siRNA/WS (1 or 10 μg) or control siRNA/WS (10 μg) was injected intravenously in CIA mice three times per week starting from the day of the second immunization (day 0) to day 10. The incidence of arthritis (A), arthritis score (B), and paw thickness (C) were evaluated continuously until day 10. D, at day 12, TNF-α mRNA expression in the paw was analyzed by real-time RT-PCR. Data are the mean ± S.E.M. of 5 to 15 mice in each group. * P < 0.05 mice treated with TNF-α siRNA/WS 10 μg versus control siRNA/WS. Bar, control siRNA/WS; 10 μg/body; ○, TNF-α siRNA/WS, 1 μg/body; ●, TNF-α siRNA/WS, 10 μg/body; †, normal.
is designed to maximize enhanced permeability and retention effect (Hall et al., 2007). In addition, we recently found that systemically injected siRNA/WS was accumulated in 12-O-tetradecanoylphorbol-13-acetate-treated skin area in a mouse model of skin inflammation (N. Yagi, unpublished observations). These data also indicate that the siRNA/WS has the nature of accumulating in the inflamed tissue, probably due to an increase in vascular permeability. As an alternative, activated macrophages and neutrophils in the inflamed synovium may have enhanced siRNA/WS uptake activity. Further studies are needed to examine these possibilities and to determine the mechanism of action of siRNA/WS. The siRNA/WS system could potentially be applicable to other systemic diseases in which macrophages play a central pathogenic role. Understanding the molecular mechanism of inflammatory tissue-selective siRNA/WS delivery should help us further improve the therapeutic effect of WS-mediated siRNA delivery.

There are only a few studies that reported the success of siRNA-based therapy for RA in animal models (Inoue et al., 2005; Schiiffelers et al., 2005; Khoury et al., 2006, 2008). Khoury et al. (2006, 2008) reported that systemic administration of a cationic liposome carrying siRNA directed against TNF-α or a cocktail of siRNAs directed against pro-inflammatory cytokines, including IL-1, -6, and -18, was effective against CIA. However, the cationic liposome used in the above studies could rapidly be distributed to reticulendothelial systems because of the micron-sized diameter and positive surface property. In contrast, siRNA/WS was designed to be nanosized (100 nm in diameter) and neutrally charged, resulting in its efficient distribution into peripheral inflamed tissue (Yagi et al., 2009). Indeed, we confirmed remarkable accumulation and cellular incorporation of the siRNA-encapsulated WS in inflamed synovium; thus, the relative contribution of macrophages in the affected joints to the therapeutic effect of siRNA might be higher in the WS than in the previously reported cationic liposome.

Treatment with TNF-α siRNA/WS from day 0 (when clinical arthritis just started developing) showed a therapeutic effect in CIA. However, when we started the treatment from day 7 (when the arthritis were established), the CIA was not suppressed (data not shown). Combining siRNA/WS with a specific targeting approach (e.g., surface modification of liposome) may improve the therapeutic efficacy of this system (Koning et al., 2006).

In conclusion, our results showed that the siRNA/WS system enabled efficient delivery of siRNA to arthritic joints. The siRNA/WS was incorporated into CD11b+ macrophages and neutrophils in the inflamed synovium, suggesting that it could be a therapeutic tool to silence the expression of various molecules associated with the pathogenesis of RA produced by these cells.

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Authorship Contributions

Participated in research design: Komano, Miyasaka, and Nanki.

Conducted experiments: Komano, Yagi, Onoue, and Kaneko.

Contributed new reagents or analytic tools: Yagi.

Performed data analysis: Komano, Miyasaka, and Nanki.

References


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