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Small interfering RNA targeting CD81 ameliorated arthritis in rats

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ABSTRACT

CD81 belongs to a family of cell-surface protein (tetraspanin) known as one of the up-regulated elements in rheumatoid arthritis synoviocytes. In this study, the therapeutic effect of small interfering RNA targeting CD81 (siCD81) was examined by *in vivo* electroporation method. Treatment with siCD81 significantly ameliorated paw swelling of collagen-induced arthritic (CIA) rats. In histological examination, hypertrophy of synovium, bone erosion, and degeneration of articular cartilage were milder in rats treated with siCD81 than in the control group and the non-specific siRNA group. Expression of synoviolin, a rheumatoid regulator, was suppressed by siCD81. Thus, therapeutic intervention by targeting CD81 may be used in the treatment of rheumatoid arthritis.

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Introduction

Rheumatoid arthritis (RA) is one of the most common articular diseases, characterized by hyperproliferation of synovial cells and bone destruction. The causes of RA are multifactorial, involving genetic, stress, hormonal, and immunological factors that are all important in development [1,2]. Recently, biological agents that hinder the function of inflammatory cytokine such as TNF-α and IL-6 have been clinically developed to treat RA with a more effective suppression of joint inflammation than conventional anti-rheumatic agents [3,4]. As these agents can cause infections like tuberculosis, as well as being costly, further investigations are needed to achieve remission of arthritis [5].

The E3 ubiquitin ligase synoviolin, or 3-hydroxy-3-methylglutaryl reductase degradation (Hrd1), is a rheumatoid regulator highly associated with the development of RA [6,7]. Its inhibition may have therapeutic benefits in the prevention or treatment of RA. However, the ligands or receptors involved in overexpression of synoviolin have not been elucidated.

CD81 belongs to a family of cell-surface protein known as tetraspanin. It influences adhesion, morphology, activation, proliferation, and differentiation of B and T cells [8,9]. Our previous study on gene profiling of RA synoviocytes using genome-wide DNA chips found that CD81 is involved with proteins up-regulated in RA synoviocytes *in vitro* [10]. Thus, we analyzed the relationship between synoviolin expression and the activation of CD81. To determine whether inhibition of CD81 may be used in a therapeutic treatment of RA, we examined the therapeutic effect of small interfering RNA (siRNA) targeting CD81 by an *in vivo* electroporation method.

Materials and methods

Animals and induction of collagen-induced arthritis (CIA). Dark Agouti (DA) rats were purchased from Shimizu Laboratory Suppliers (Kyoto, Japan). Animal experiments were conducted according to the Guidelines regarding Animal Research of the Kyoto Prefectural University of Medicine. To induce CIA, collagen type II (Collagen Research Center, Tokyo, Japan) was dissolved in 0.01 M acetic acid (2 mg/ml) and emulsified 1:1 in Freund's incomplete adjuvant (Sigma) (CII/FIA) on ice. DA rats weighing 200–250 g were intradermally injected with 200 μl CII/FIA solution at the base of the tail [11].

siRNA duplexes.

Three siRNA duplexes targeting the rat CD81 gene were synthesized (A: 5′-CAACUCAUUCACUCAGCUUTT-3′ and 5′-AAGCUGAGUGAAUGAGUUGTT-3′; B: 5′-GUAGCUGUCAUUAUGAUCUTT-3′ and 5′-AGAUCAUAAUGACAGCUACTT-3′; C: 5′-CUGUCAUUAUGAUCUUCGATT-3′ and 5′-UCGAAGAUCAUAAUGACAGTT-3′). Experiments were conducted *in vitro* to most effective siRNA sequences for suppression of CD81 expression in rats. Each siRNA was transfected

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into cultured synovial fibroblasts from rats to compare silencing competence; synovial fibroblasts were obtained from rats as previously described [12]. To transfect siRNA, synovial fibroblasts (2×10^5 cells/well) were seeded onto 6-well plates (Iwaki Glass, Chiba, Japan). After 24 h, the culture medium was aspirated, and 1.9 ml fresh complete medium was added. A mixture of siRNA (200 pmol), 8 μl RNAiFect Transfection Reagent (Qiagen, Hilden, Germany), and 90 μl Buffer EC-R (Qiagen) was dropped onto each well. After 24 h, total RNA was extracted using Sepasol-RNA super II (Nacalai Tesque) and then purified with RNeasy mini kit (Qiagen). Quantitative real-time PCR was performed in an ABI PRISM 7000 sequence detection system using a TaqMan gene expression assay primer, probe sets of rat CD81 and GAPDH, and gene expression master mix (Applied Biosystems). CD81 gene expression was normalized using the internal control gene GAPDH. Most potent siRNA was used for the following examinations. AllStars Negative Control siRNA (Qiagen) was used as non-specific siRNA.

Electroporation-assisted siRNA transduction in vivo. Rats were anesthetized by an intraperitoneal injection of 1 μl/g sodium pentobarbital, and 50 μl siRNA/siPORT Amine (Ambion) complex containing 800 pmol siRNA and 10 μl siPORT Amine was administered into the left knee joint using a 27-gauge needle. Immediately after the injection, keratin cream (Fukuda Denshi, Tokyo, Japan) was coated around the knee joint, onto which a pair of electrode pads (1.0 cm in diameter) was placed. Using a CUY21 electric pulse generator (NeppaGene, Tokyo, Japan), three square-wave pulses with a pulse length of 100 ms were loaded at 150 V/cm at a frequency of 1 s^{-1} followed by two other pulses with the opposite polarity [13].

Evaluation of the silencing effects of siRNA in vivo. To analyze CD81 mRNA expression *in vivo*, the synovium was removed from the region surrounding the left patella of rats 24 h after electroporation-assisted siRNA transduction. We used synovium from rats which was administered non-specific siRNA as control. Total RNA was extracted using Sepasol-RNA super II and then purified with RNeasy mini kit. Quantitative real-time PCR was performed as above mentioned. CD81 gene expression was normalized using the internal control gene GAPDH.

Evaluation of CIA rats. CIA rats were immunized according to the above method. Each siRNA was transfected every 3 days (i.e., 7, 10, 13, and 16 days after immunization). After electro-transduction of siRNA, foot volume was measured using a water replacement plethysmometer (Unicom Japan, Tokyo, Japan) [14]. The cumulative percentage of rats exhibiting ankle swelling on the lower limb of the treated side was calculated to determine the chronology of the onset of CIA. After 28 days post-immunization, radiographs of the hind paw of CIA rats were obtained using Technomobile II (Hitachi, Tokyo, Japan) at 50 kV/2.0 mAs, at a focus-skin distance of 100 cm.

Histological analysis. The left knee and ankle joints of CIA rat were excised 28 days after immunization and fixed in 4% paraformaldehyde. Following decalcification with EDTA, 6-μm thick sagittal sections were prepared from the center of the lateral condyle of the femur, as well as from the center of the foot joint. The sections were then stained with hematoxylin and eosin (H&E) or Safranin O. Arthritic changes, such as infiltration of inflammatory cells, synovial proliferation, destruction of articular cartilage, and bone erosion, were evaluated 28 days after immunization using histological scores as described [15]. To evaluate the immunohistochemistry of synoviolin, endogenous peroxidase activity was blocked by incubating the sections in 0.3% H_2O_2 in methanol for 30 min. Antigen retrieval was achieved by heat treatment using 10 mM citrate buffer solution (pH 6.0). After treatment with normal goat serum, the sections were incubated at 4 °C with anti-synoviolin (kindly provided by Dr. Toshihiro Nakajima, St. Marianna University) at 1:500 overnight. Tagging of primary antibody was achieved by subsequent application of Mouse IgG ABC kit (Vectastain Elite ABC kits, Vector Laboratories, USA). Visualization of immunohistochemical reaction was performed by developing the enzyme complex with DAB/H_2O_2 solution (Histofine DAB substrate; Nichirei, Japan) and counterstained with Mayer's hematoxylin.

Statistical analysis. All data were reported as the mean ± standard deviation (SD). The data were analyzed by analysis of variance (ANOVA). Post hoc testing was performed using Tukey–Kramer test. Nonparametric Mann–Whitney *U* test was used to evaluate the statistical significance of differences in histological scores. *P* values less than 0.05 were considered to indicate statistical significance.

Results

Gene silencing effect of CD81-specific siRNA (siCD81) in vitro and in vivo

Three rat siCD81 duplexes (designated as A, B, and C) were synthesized. Although significant gene silencing of CD81 was induced by all three siRNAs, the most potent effects were observed with siCD81-C, with successful reduction in gene expression in the cultured synovial fibroblast (Fig. 1A). Thus, siCD81-C was used for the following experiments. We measured CD81 gene expression from the synovium of rats using real-time PCR to reveal *in vivo* silencing effect of siCD81-C. The expression of CD81 in the synovium of the knee joints was reduced compared that of control joints (Fig. 1B).

Therapeutic effect of siCD81 in CIA rats

We assessed whether the delivery of siCD81 in the joints suppressed the clinical manifestations of RA in rats. Each siRNA was

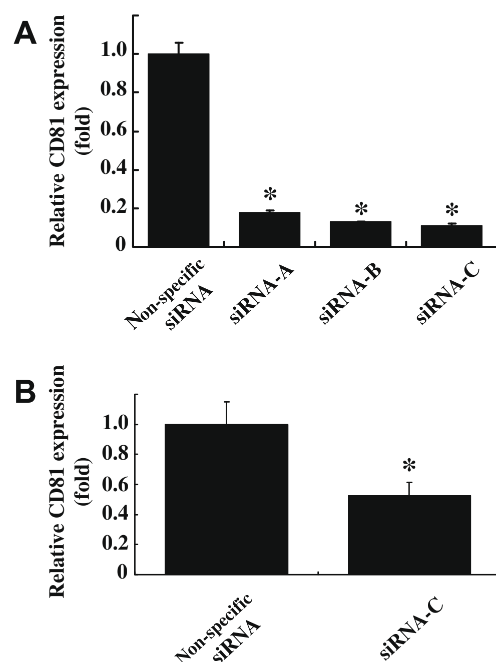

Fig. 1. Suppression of CD81 mRNA *in vitro* and *in vivo*. (A) Three siRNA duplexes specific for rat CD81 were transfected in synovial fibroblast derived from DA rats, while non-specific siRNA was transfected as the control. (B) Synovium were obtained from knee joint of DA rats 24 h after electroporation-assisted siRNA transduction. The relative expression of CD81 was examined by real-time PCR. *$P < 0.05$, versus non-specific siRNA.

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