RING-finger type E3 ubiquitin ligase inhibitors as novel candidates for the treatment of rheumatoid arthritis

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Abstract. Rheumatoid arthritis (RA) significantly affects quality of life. We recently cloned synoviolin, a RING-type E3 ubiquitin ligase implicated in the endoplasmic reticulum-associated degradation (ERAD) pathway. Synoviolin is highly expressed in rheumatoid synovial cells and may be involved in the pathogenesis of RA. Inhibition of synoviolin activity is a potentially useful therapeutic approach for the treatment of RA. We conducted a high-throughput screen of small molecules to find inhibitors of synoviolin autoubiquitination activity. We identified two classes of small molecules, named LS-101 and LS-102, which inhibited synoviolin activity. LS-102 selectively inhibited synoviolin enzymatic activity, while LS-101 inhibited a broad array of RING-type E3 ligases. Moreover, these inhibitors suppressed the proliferation of rheumatoid synovial cells, and significantly reduced the severity of disease in a mouse model of RA. Our results suggest that inhibition of synoviolin is a potentially useful approach in the treatment of RA.

Introduction

Rheumatoid arthritis (RA) is the most common chronic inflammatory joint disease, affecting ~0.5-1% of people in the industrialized world (1). Clinically, the disorder is characterized by joint pain, stiffness, and swelling due to synovial inflammation and effusion. The clinical features of RA are based on several pathological processes including chronic inflammation, overgrowth of synovial cells, bone and joint destruction, and fibrosis. Currently, the goal of RA treatment is the control of underlying inflammatory process to prevent joint damage using non-steroidal anti-inflammatory drugs, glucocorticoids, and disease-modifying anti-rheumatic drugs (DMARD). The most widely used small molecule DMARD is methotrexate, which shows the highest retention rate compared with other agents (2). In recent years, biological agents such as inhibitors of tumor necrosis factor (TNF) signaling have become available for clinical use; however, this therapy is prohibitively expensive, and although TNF inhibitors are clinically as effective as methotrexate, the frequency and extent of response are more restricted. In fact, many patients can lose the clinical response to TNF inhibition, highlighting the need for other treatment modalities to further improve the outcome of RA (3,4).

To address this need, we have been investigating the mechanism of outgrowth in rheumatoid synovial cells (RSCs). First, we demonstrated the crucial role of Fas antigen-induced apoptosis in synovial cell hyperplasia (5). Then, while studying cellular functions of RSCs, we cloned synoviolin from these cells (6). Synoviolin, a mammalian homolog of Hrd1p/Der3p (7-9), is an endoplasmic reticulum (ER)-resident E3 ubiquitin ligase with a RING motif that is involved in ER-associated degradation (ERAD) pathway. Synoviolin is also highly expressed in synoviocytes of patients with RA (6,10-12). Overexpression of synoviolin in transgenic mice leads to advanced arthropathy caused by reduced apoptosis of synoviocytes (6). We postulated that hyperactivation of the ERAD pathway by overexpression of synoviolin prevents ER-stress-induced apoptosis, leading to synovial hyperplasia (13). Synoviolin$^{+/-}$ knockout mice showed resistance to the development of collagen-induced arthritis (CIA) due to enhanced apoptosis of synovial cells (6). Consistent with our hypothesis, cells from these mice show impaired ERAD due to the lack of synoviolin. In addition, synoviolin ubiquitinates and sequesters the tumor suppressor p53 in the cytoplasm, thereby negatively regulating its biological functions in transcription, cell cycle regulation, and apoptosis by targeting it instead for proteasomal degradation (14). Therefore, synoviolin regulates apoptosis in response to ER stress (through ERAD) as well as p53-dependent apoptosis.

Together, these studies implicated synoviolin as a candidate pathogenic factor in arthropathy, and suggested that the gene dosage of this protein correlates with the onset of arthropathy. Furthermore, elevated synoviolin levels were identified in

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circulating monocytes in association with resistance to treatment with infliximab (a monoclonal antibody against TNF) (10). Therefore, blocking the function of synoviolin could be clinically beneficial in RA patients. This study attempted to identify an inhibitor of synoviolin that acts by blocking its enzymatic activity.

Materials and methods

Screening of synoviolin inhibitor. Purified glutathione S-transferase (GST)-synoviolin Δ transmembrane domain (TM) was mixed with glutathione-SPA beads (Amersham Pharmacia Biotech) in buffer (50 mM Tris-HCl, pH 7.4, Protease inhibitor cocktail, 14 mM β-mercaptoethanol, 0.5 μl cell lysate/well, 0.2 mg SPA bead/well) and incubated for 30 min at room temperature. Glutathione-SPA beads were washed twice, and then mixed with the candidate synoviolin inhibitor compounds in buffer (50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 2 mM NaF, and 10 nM okadaic acid) in the presence of ATP (2 mM), ^{33}P-labeled ubiquitin (0.38 μg/well), E1 (25 ng/well) (Affiniti Research), and E2 (0.3 μg/well) (UbcH5c). After incubation for 90 min at room temperature, buffer comprising 0.2 M boric acid, pH 8.5, 2 mM ethylenediaminetetraacetic acid (EDTA), and 2% Triton-X100 was added to stop the reaction. The beads were allowed to settle and the amount of ^{33}P-ubiquitin incorporated into the GST-synoviolin beads was determined using a Microbeta Scintillation counter.

The primary screen was conducted with multiple compounds per well (10-20 compounds per well) at an estimated screening concentration of 2-10 μM. Compound mixtures showing potential activity in the primary screen were then rescreened at one compound per well to determine the active compound within the mixture. Three equivalents of a single compound per well follow-up screening were evaluated. Reconfirmed active compounds were resynthesized and tested in a dose-response experiment to determine potency.

In vitro ubiquitination assay. The *in vitro* ubiquitination assay used in this study was described previously (15). Briefly, 40 ng of E1 (Affiniti Research), 0.3 μg of E2 (UbcH5c), 0.75 μg of ^{32}P-labeled ubiquitin (a gift from T. Ohta), and 1 μg of recombinant E3 ubiquitin ligases were incubated for 30 min at 37˚C. Samples were analyzed as described above.

Cells. HeLa cells were obtained from ATCC. Synovial cells were isolated from synovial tissue obtained patients with rheumatoid arthritis (RA) who met the American College of Rheumatology criteria for RA at the time of orthopedic surgery. These cells were cultured in Dulbecco's modified Eagle's medium (Sigma).

Proliferation assay. The proliferation of rheumatoid synovial cells (RSCs) was evaluated using Alamar blue (BioSource International) according to the manufacturer's instructions.

Induction of CIA. CIA was induced as described previously (6). Briefly, bovine type II collagen (Collagen Research Center) was dissolved overnight in 0.05 M acetic acid at 4˚C, and then emulsified in complete Freund's adjuvant (Difco) to a final concentration 1 mg/ml. DBA/1 male mice (7-week-old) were immunized by subcutaneous injections containing 100 μg of collagen emulsion. After 3 weeks, mice were boosted with 200 μg collagen emulsion in Freund's complete adjuvant. Then, the mice were treated daily for 4 weeks with the inhibitor compounds at 1.3, 4.0, and 12.0 mg/kg/day in olive oil, vehicle control intraperitoneally, or oral administration of 0.25 mg/kg/day dexamethasone in methylcellulose as a positive control.

The mice were monitored daily for signs of arthritis using an established scoring system (16): 0, no swelling or redness; 1, swelling, redness of paw or 1 joint; 2, two joints involved; 3, more than two joints involved; 4, severe arthritis of entire paws and joints. All paws were evaluated in each animal and the maximum score per animal was 16.

Histological studies. The knee and elbow joints were fixed in 4% paraformaldehyde. After decalcification with EDTA, the joints were embedded in paraffin, and 4-μm sections were prepared for staining with hematoxylin and eosin. The extent of arthritis in the joints was assessed according to the method reported by Tomita *et al* (17): 0, normal synovium; 1, synovial membrane hypertrophy and cell infiltration; 2, pannus and cartilage erosion; 3, major erosion of cartilage and subchondral bone; 4, loss of joint integrity and ankylosis.

Statistical analysis. All data are expressed as mean ± SEM. Differences between groups were examined for statistical significance using Student's t-test. A P-value <0.05 denoted the presence of a statistically significant difference.

Ethical considerations. The ethics committee for Animal Experiments of St. Marianna University School of Medicine approved the mice experiments described in this study. Furthermore, all the experimental protocols described in this study were approved by the Ethics Review Committee of St. Marianna University School of Medicine (Approval number 01008), and the written informed consent was obtained from all patients.

Results

High-throughput compound screening for inhibitors of synoviolin. To identify small molecule inhibitors of synoviolin autoubiquitination, we screened the Lead Discovery Service program of Pharmacopeia, which includes more than four million compounds from Pharmacopeia's Compound Collection (18). Herein we monitored ^{33}P-autoubiquitinated synoviolin in cell lysates containing GST-synoviolinΔTM in the presence of ATP, E1, E2, and ^{33}P-labeled ubiquitin (Fig. 1A). The primary screen was conducted with multiple compounds per well (10-20 compounds per well) at an estimated screening concentration of 2-10 μM. Mixtures of compounds showing potential activity in the primary screen were then rescreened individually. Compounds demonstrating activity in this reconfirmation assay were resynthesized and retested. Two unique compounds, termed LS-101 and LS-102, inhibited the autoubiquitination of synoviolin with a 50% inhibitory concentration value (IC_{50}) of ~15 μM (Fig. 1B) and 20 μM (Fig. 1C), respectively.

LS-101 and LS-102 inhibit the autoubiquitination of synoviolin. Further evaluation of LS-101 and LS-102 in an *in vitro* ubiqui-

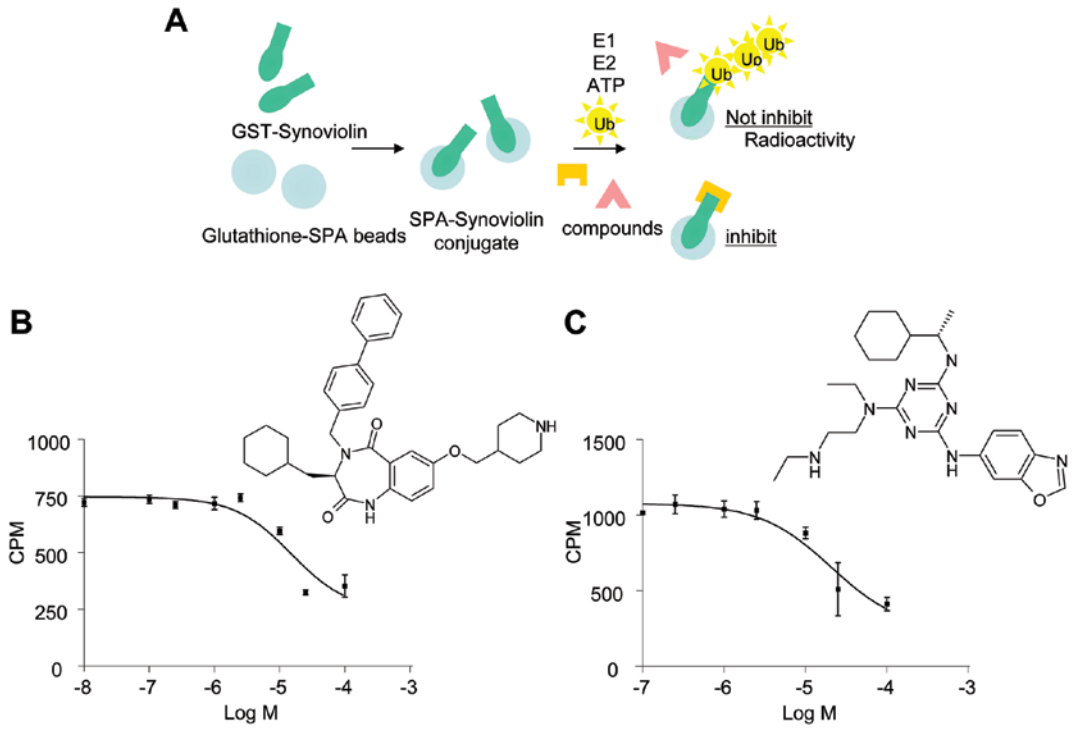

Figure 1. Screening for synoviolin inhibitors. (A) Scheme of high-throughput screening of synoviolin-induced ubiquitination assay. (B) Inhibition of synoviolin ^{33}P-polyubiquitination by LS-101 and chemical structure of LS-101. (C) Inhibition of synoviolin ^{33}P-polyubiquitination by LS-102 and chemical structure of LS-102.

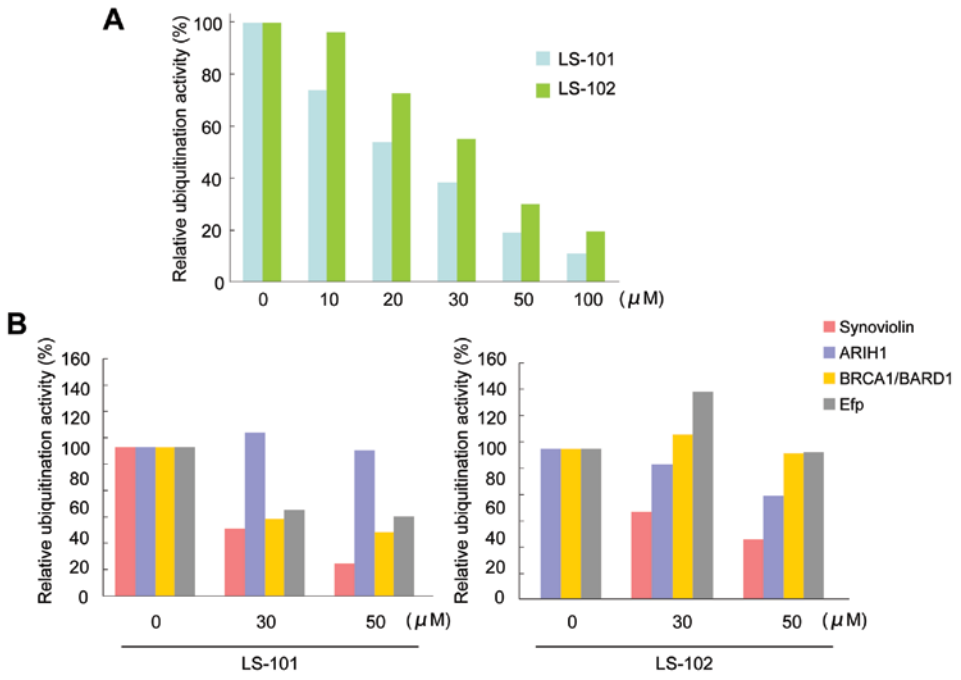

Figure 2. Effects of LS-101 and LS-102 on *in vitro* ubiquitination. (A) Both LS-101 and LS-102 inhibited the autoubiquitination of synoviolin in a dose-dependent manner. The IC_{50} of LS-101 was 20 μM and that of LS-102 was 35 μM. (B) Selectivity of LS-101 (left) and LS-102 (right) against other E3 ubiquitin ligases. LS-102 inhibited synoviolin selectively compared with LS-101. Data are mean ± SEM of 3 experiments.

tination assay showed that the inhibition of synoviolin activity by both LS-101 and LS-102 was dose-dependent (LS-101; IC_{50}=20 μM, LS-102; IC_{50}=35 μM) (Fig. 2A). To assess the selectivity of the compounds for other E3 ubiquitin ligases, we determined the effects of LS-101 and LS-102 on the enzymatic activity of the following RING-finger type E3 ubiquitin ligases: ariadne, *Drosophila*, homolog of, 1 (ARIH1) (19), breast cancer 1 gene (BRCA1)/BRCA1-associated RING domain 1 (BARD1) (20), and estrogen-responsive RING-finger protein (Efp) (21). LS-101 inhibited the activity of BRCA1/BARD1 and Efp

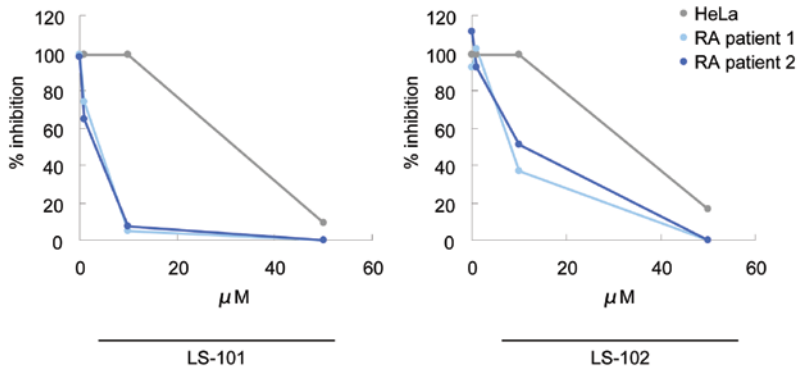

Figure 3. Effects of LS-101 and LS-102 on cell growth of RSCs. HeLa cells and RSCs derived from two RA patients were treated with synoviolin inhibitors for 12 h at the indicated concentrations. LS-101 and LS-102 repressed the proliferation of each RSC population tested. Data are expressed as the mean percentage of inhibition of the vehicle-treated control group ± SEM; (n=3).

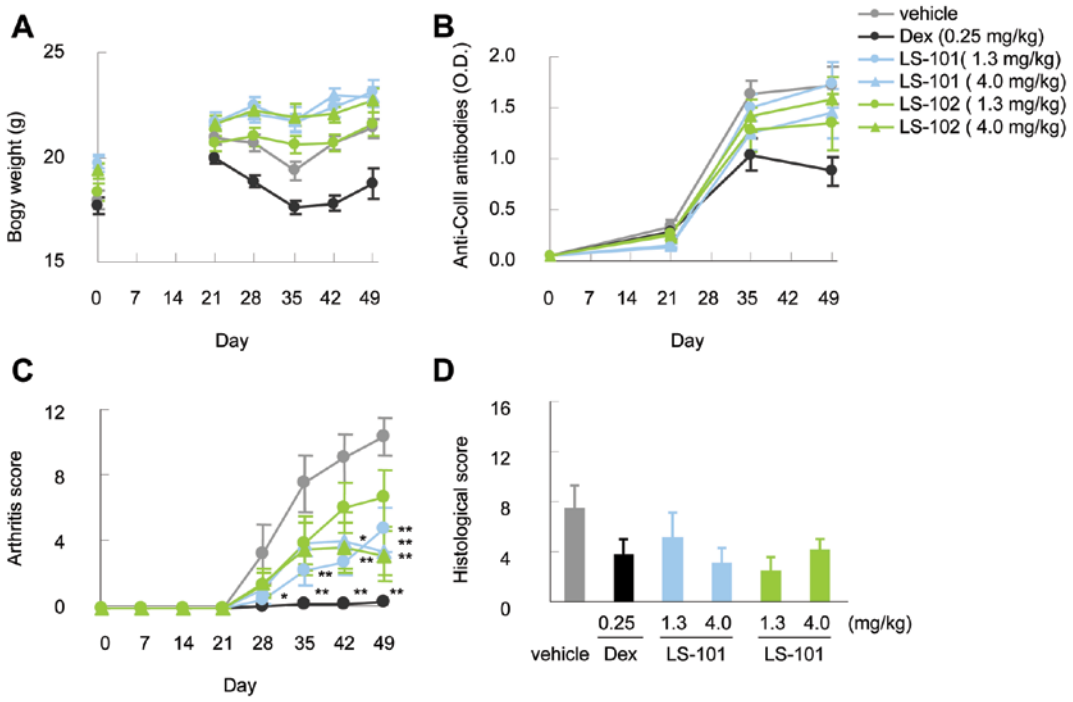

Figure 4. Effects of LS-101 and LS-102 in mouse CIA. DBA/1 mice immunized on day 0 and boosted on day 21 with type II collagen were treated with the vehicle alone, 0.25 mg/kg dexamethasone (Dex), or with 1.3, 4.0 mg/kg LS-101 or LS-102 from day 21 to 49. (A) Change in body weight. (B) The level of anti-type II collagen antibodies. (C) Total arthritis score. (D) Histological arthritis score. Data are mean ± SEM (initial n=12; final n=7). *P<0.05, **P<0.01.

(Fig. 2B), although this effect was weaker than that observed with synoviolin (Fig. 2B). Moreover, LS-101 had no effect against the enzymatic activity of ARIH1 (Fig. 2B). On the other hand, LS-102 did not inhibit the activity of other E3 ubiquitin ligases, only affecting synoviolin (Fig. 2B). These results suggested that LS-102 is a more selective synoviolin inhibitor than LS-101.

LS-101 and LS-102 inhibit proliferation of RSCs. We next tested LS-101 and LS-102 for their effects on the proliferation of RSCs, using HeLa cells as a control. LS-101 and LS-102 inhibited HeLa cell growth only at very high concentrations (LS-101; IC_{50}=31.3 μM, LS-102; IC_{50}=32.7 μM). However, treatment of RSCs with these compounds suppressed synovial cell growth dose-dependently and with much greater potency than that observed in HeLa cells (Fig. 3). A similar effect was also observed in another line of RSCs (Fig. 3). In addition, LS-101 inhibited synovial cell proliferation more potently than LS-102 (LS-101; IC_{50}=4.2 μM, LS-102; IC_{50}=5.4 μM). These results demonstrated that blockade of synoviolin function reduced the proliferation of RSCs, and that RSCs are more susceptible to this effect than HeLa cells. Consistent with these findings, higher expression levels of synoviolin were observed in RSCs than in HeLa cells (6).

LS-101 and LS-102 reduce clinical severity scores in a CIA model. To evaluate the *in vivo* efficacy of synoviolin inhibitors, we tested LS-101 and LS-102 in a mouse model of arthritis over a period of 28 days. No reduction of body weight was observed during the administration of these compounds (Fig. 4A). Moreover, the production of anti-type II collagen antibodies resulting from type II collagen immunization in both the LS-101 and LS-102 group was comparable to that

observed in the vehicle control group (Fig. 4B). Intraperitoneal treatment with LS-101 or LS-102 starting on day 21 reduced the clinical severity scores compared to vehicle controls (Fig. 4C). The efficacy was observed at both 1.3 mg/kg and 4.0 mg/kg doses in this experiment, although the protective effect of LS-101 at 1.3 mg/kg against CIA was stronger than the same dose of LS-102. At 4.0 mg/kg, there was no difference in the effects between LS-101 and LS-102. Finally, histological analysis showed lower histological arthritis scores in mice treated with the synoviolin inhibitors compared with wild-type mice (Fig. 4D).

Discussion

The selective degradation of proteins in eukaryotic cells is carried out by the ubiquitin proteasome system (UPS), whereby proteins are targeted for degradation by covalent ligation to small polypeptide ubiquitin (22,23). This reaction requires the sequential actions of three enzymes: E1, E2, and E3 ligases (22,23). E3 ligases are responsible for conferring selectivity to ubiquitination by recognizing specific substrates. Bioinformatic analysis has identified over 600 E3 ligases, with RING-type E3 ligases constituting the largest subfamily within this group (24). Accordingly, RING E3 ligases have been linked to the control of multiple cellular processes and to many human diseases such as diabetes mellitus, polyglutamine disease, and Parkinson's diseases (24-26). In the UPS, the proteasome inhibitory agent bortezomib (Velcade) was recently approved for the treatment of multiple myeloma and mantle cell lymphoma (27). Bortezomib induces apoptosis of a wide variety of cancer cells, and is the first proteasome inhibitor to gain FDA approval (28-30). However, widespread clinical use of bortezomib continues to be hampered by the appearance of dose-limiting toxicities, drug-resistance, and interference by some natural compounds (31). Thus, despite the efficacy of bortezomib for treating lethal diseases such as cancer, the associated toxicities prevent its use for the treatment of chronic diseases such as RA. Thus, it is important to develop inhibitors of the ubiquitin-proteasome enzymatic cascade upstream from the proteasome to impact fewer cell processes and reduce toxicity. E3 ligases are attractive such targets given their large number and substrate specificity. We recently cloned the E3 ubiquitin ligase synoviolin, which localizes to the ER lumen and has enzymatic activity. We have also demonstrated that this protein plays crucial roles in the pathological processes of RA (6), and could therefore be a candidate novel therapeutic target of RA (32).

In this study, we identified two potent small compounds as inhibitors of synoviolin enzymatic activity using high-throughput screening (Fig. 1). Moreover, *in vivo* studies showed no serious toxicity associated with these compounds in terms of survival and weight loss during treatment (Fig. 4A). Biochemical characterization of the two compounds, LS-101 and LS-102, demonstrated that they both inhibit the autoubiquitination activity of synoviolin *in vitro* (Fig. 2), with LS-101 showing stronger efficacy (IC_{50}=20 μM) than LS-102 (IC_{50}=35 μM), but less selectivity (Fig. 2). It was unclear from this study why LS-101 showed a weak inhibitory effect on BRCA1/BARD1 and Efp activity, and further study is needed to understand the molecular basis for this observation. LS-101 and LS102 inhibited the proliferation of RSCs and to a much lesser extent, HeLa cells (Fig. 3). The difference in cell sensitivities to these compounds could be, at least in part, due to the expression level of synoviolin, namely, high levels of synoviolin in RSCs would contribute to the cell overgrowth and therefore, inhibition of synoviolin in these cells would in turn suppress proliferation. These cells may also have different requirements for synoviolin, such that repressing synoviolin activity in RSCs would lead to growth suppression. Prophylactic administration of either LS-101 or LS-102 also significantly reduced the severity of murine CIA (Fig. 4C). Since LS-101, a nonselective inhibitor, reduced clinical severity scores in CIA similarly to LS-102, blocking synoviolin enzymatic activity seems crucial in the pathological process of CIA. These findings suggest that the suppression level of synovial cell growth and incidence of arthritis reflect the efficacy of these compounds rather than their selectivity, and that in RA, synoviolin might have an indispensable role among E3 ligases.

RA comprises multiple processes such as chronic inflammation, overgrowth of synovial cells, joint destruction, and fibrosis. During the course of inflammation, synovial cells, macrophages, T cells, and B cells all contribute to the production of cytokines such as interleukin (IL)-1, IL-6, IL-10, TNF, and transforming growth factor β (TGF-β) (33,34). These cytokines, in turn, stimulate the overgrowth of synovial cells to form a mass of synovial tissue, called pannus, which invades and destroys the bone and cartilage through osteoclast activation and protease production (33-37). This chronic inflammation state ultimately leads to fibrosis. Our study proved that synoviolin is, at least in part, involved in the overgrowth of synovial cells (6) and fibrosis (38) among these processes. The IL-17 induction of synoviolin may also contribute to RA chronicity (39), and synoviolin has been shown to target misfolded MHC class I heavy chains (40). In this study, antibody titers were elevated in synoviolin inhibitor-treated mice to levels comparable to those in vehicle controls (Fig. 4B). Thus, as with the study of synoviolin$^{+/-}$ knockout mice in CIA, it is difficult to clarify the function of synoviolin with respect to the chronicity of inflammation, because suppressing synoviolin blocks synovial cell outgrowth directly due to sequential events following immunization of type II collagen (6). Our results confirm that further studies of the association between chronic inflammation and synoviolin are clearly warranted.

Eight biological agents are currently approved for clinical use in treatment of RA, and these drugs have dramatically changed the outcome of RA during the past decade (3,4). However, some patients still fail to respond to the biological treatment or develop adverse effects such as an increased risk of infection. Moreover, these agents are associated with high costs and discomfort arising from the subcutaneous or intravenous administration. Thus, there is a clear need for the development of cheaper, orally administered therapies with fewer side effects. In this regard, spleen tyrosine kinase (Syk) inhibitor, an orally administered drug, has been developed for the treatment of RA (41,42). Dual blockade of TNF and IL-17 was also reported recently as a strategy for halting RA disease from progression to the extent seen when only one cytokine is blocked (43). The involvement of synoviolin in both the TNF and IL-17 pathways further implicates inhibitors of this enzyme as potential candidate drugs for treatment of RA.

In conclusion, we identified two strong synoviolin inhibitors, and confirmed that synoviolin is an ideal molecular target for RA for disease modification and treatment. We are now proceeding with the optimization of LS-101 and LS-102, and hope our research will lead to the development of a new therapy for RA.

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ORIGINAL ARTICLE

Serum level of soluble triggering receptor expressed on myeloid cells-1 as a biomarker of disease activity in relapsing polychondritis

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Abstract
Objectives We aimed to identify a serum biomarker for evaluating the disease activity of relapsing polychondritis (RP).
Methods We measured and compared serum levels of 28 biomarkers potentially associated with this disease, including soluble triggering receptor expressed on myeloid cells-1 (sTREM-1), high-sensitivity C-reactive protein (hs-CRP), and cartilage oligomeric matrix protein (COMP), in 15 RP patients and 16 healthy donors (HDs). We divided the 15 RP patients into active RP ($n = 8$) and inactive RP ($n = 7$) groups, depending on the extent of the disease, and compared candidate markers between groups. The localization of membrane-bound TREM-1 in the affected tissue was examined by immunohistochemistry.
Results Serum levels of sTREM-1, interferon-γ, chemokine (C–C motif) ligand 4, vascular endothelial growth factor, and matrix metalloproteinases-3 were significantly higher in RP patients than HDs. Among these markers, sTREM-1 had the highest sensitivity and specificity (86.7 and 86.7 %, respectively). Furthermore, the serum level of sTREM-1 was significantly higher in active RP patients than inactive RP patients ($p = 0.0403$), but this was not true for hs-CRP or COMP. TREM-1 was expressed on endothelial cells in RP lesions.
Conclusions The serum level of sTREM-1 may be a useful marker of disease activity in RP.

Keywords Relapsing polychondritis · Serum marker · Soluble triggering receptor expressed on myeloid cells-1

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Introduction

Relapsing polychondritis (RP) is a rare inflammatory disorder of unknown etiology; it is characterized by recurrent, widespread chondritis of systemic cartilages, specifically those in the ear, eye, nose, large airways, and joints [1–3]. RP is occasionally life-threatening, as its progression leads to fatal dyspnea due to cartilage destruction in large airways. To detect such disease progression, the accurate assessment of disease activity is important. Today, this assessment is performed by analyzing a combination of clinical manifestations, laboratory findings, and imaging results.

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However, it is still difficult to conduct proper evaluations. This is partly because there are no established biomarkers for evaluating the disease activity of RP, although several potential biomarkers—such as CRP, antibody to type II collagen, and cartilage oligomeric matrix protein (COMP)—have been reported previously [3–7]. For example, CRP is the most commonly used marker of inflammation, and its serum level is frequently used to assess RP disease activity [3, 4]. However, RP patients with normal CRP levels are often observed to experience advanced fibrosis of the airways, suggesting insidious chronic inflammation in those tissues, which is difficult to detect by CRP [8]. It has also been reported that antibodies to type II collagen reflect RP disease activity [6]. However, these antibodies were only detected in 30–50 % of RP patients [6, 9]. Furthermore, it has been reported that this measure lacks sensitivity and specificity [10]. Therefore, in the current study, we aimed to identify more sensitive biomarkers that would be able to detect those small differences that cannot be detected by antibodies to type II collagen or CRP.

To do so, this study excluded highly active RP patients. We measured 28 candidate markers that had been previously shown to be involved in RP, inflammation, or cartilage destruction. The levels of these markers were compared not only between RP patients and healthy donors (HDs) but also between active RP and inactive RP patients. Our results showed that the serum level of soluble triggering receptor expressed on myeloid cells-1 (sTREM-1) is most suitable as a disease-activity marker in RP.

TREM-1 is a type I transmembrane receptor of the immunoglobulin superfamily. The soluble form of TREM-1 (sTREM-1) is thought to be released from TREM-1-expressing cells by proteolytic cleavage of membrane-bound TREM-1 [11]. The serum level of sTREM-1 has been found to be elevated in patients with sepsis and has therefore been considered as a marker of microbial infection [12].

Materials and methods

Patients and samples

Fifteen patients (8 women and 7 men) diagnosed with RP according to Damiani's criteria [13, 14] and 16 healthy donors (HD) serving as age-matched and sex-matched controls (Table 1) were recruited from St. Marianna University Hospital, Kanagawa, Japan. They were enrolled between November and December 2009. In this study, we used the patient information (disease condition, disease duration, medication, etc.) obtained at the time of enrollment (Table 1). None of the patients had any other inflammatory disorders, such as overt infections or collagen diseases. To detect small differences that cannot be detected by CRP, this study enrolled RP patients in the chronic phase—not the acute phase—and further excluded patients who had highly active RP, such as those with acute respiratory failure. From among them, we divided the 15 RP patients into two groups (active RP and inactive RP) according to the definition by Lekpa et al. [7]. Briefly,

Table 1 Demographics, clinical characteristics, and medication of subjects

	HD	RP		
	($n = 16$)	Total ($n = 15$)	Active ($n = 8$)	Inactive ($n = 7$)
Demographics				
Age (years)[a]	40.5 [27–67]	47 [10–81]	50.5 [10–74]	44 [27–81]
Female sex	50.0 %	53.3 %	50.5 %	57.1 %
Clinical characteristics				
Disease duration (years)[a]		5 [1–19]	12 [4–19]	4 [1–8]
Auricular chondritis		46.7 %	62.5 %	28.6 %
Nasal chondritis		40.0 %	62.5 %	14.3 %
Laryngotracheal chondritis		66.7 %	87.5 %	42.9 %
Ear symptoms		53.3 %	87.5 %	14.3 %
Arthritis		46.7 %	75.0 %	14.3 %
Ocular inflammation		33.3 %	50.0 %	14.3 %
Medication				
Prednisolone		86.7 %	87.5 %	85.7 %
Methotrexate		33.3 %	50.0 %	28.6 %
Azathioprine		20.0 %	25.0 %	14.3 %

HD healthy donor, *RP* relapsing polychondritis

[a] Data are expressed as median [range]

patients were defined as having active RP if they were affected with chondritis involving at least two of three sites (auricular, nasal, or laryngotracheal cartilage) at the time of blood collection or if they were affected in one of these sites and also had two other manifestations, which could include ocular inflammation, audiovestibular symptoms, or seronegative inflammatory arthritis. Fourteen patients with HTLV-1-associated myelopathy (HAM), 10 with progressive systemic sclerosis (PSS), 19 with systemic lupus erythematosus (SLE), and 20 with rheumatoid arthritis (RA) also participated in this study.

All blood and cartilage samples were obtained with written informed consent and full ethical approval. The study protocol was approved by the Ethics Committee of St. Marianna University School of Medicine.

Measurement of serum levels of marker candidates

High-sensitivity CRP (hs-CRP) was determined by nephelometry using N-latex CRP II (Siemens Healthcare Diagnostics, Tokyo, Japan). Serum concentrations of sTREM-1; matrix metalloproteinases (MMP)-1, MMP-2, MMP-3, MMP-13; cartilage oligomeric matrix protein (COMP); interleukin (IL)-17A; and anti-type II collagen antibody (α-COLII Ab) were measured using commercially available ELISA kits (sTREM-1, MMP-1, and MMP-2: R&D Systems, Minneapolis, MN, USA; MMP-3: Daiichi Fine Chemical, Toyama, Japan: MMP-13: GE Healthcare, Chalfont St Giles, UK; COMP: Abnova, Taipei, Taiwan; IL-17A: Gen-Probe, San Diego, CA, USA; α-COLII Ab: Chondrex, Redmond, WA, USA). Serum concentrations of

Table 2 Serum concentrations of biomarker candidates in healthy donors and patients with RP

Biomarker candidates[a]	Units	Methods of measurement	HD ($n = 16$) Mean ± SD	RP ($n = 15$) Mean ± SD	p*
sTREM-1	pg/ml	ELISA	92.48 ± 56.45	281.87 ± 150.42	**0.0002**
IFN-γ	pg/ml	CBA	N.D.[c]	5.65 ± 6.25	**0.0035**
CCL4	pg/ml	CBA	64.38 ± 66.03	133.76 ± 68.13	**0.0075**
VEGF	pg/ml	CBA	131.03 ± 104.66	267.46 ± 187.03	**0.0212**
MMP-3	ng/ml	ELISA	35.96 ± 29.23	243.12 ± 313.50	**0.0229**
CXCL10	pg/ml	CBA	154.72 ± 91.72	229.50 ± 114.03	0.0552
CCL5	ng/ml	CBA	2.70 ± 1.43	37.66 ± 15.66	0.0582
hs-CRP	ng/ml	Nephelometry	0.04 ± 0.05	0.30 ± 0.50	0.0643
IL-17A	pg/ml	ELISA	1.17±1.52	0.33 ± 0.79	0.0673
TNF	pg/ml	CBA	N.D.[c]	0.76 ± 2.01	0.1646
IL-4	pg/ml	CBA	N.D.[c]	0.80 ± 2.13	0.1671
IL-6	pg/ml	CBA	N.D.[c]	1.27 ± 3.38	0.1686
COMP	ng/ml	ELISA	14.38 ± 4.28	24.33 ± 26.72	0.1750
MMP-13	ng/ml	ELISA	0.31 ± 0.04	0.28 ± 0.09	0.2367
MMP-2	ng/ml	ELISA	125.01 ± 10.45	133.01 ± 28.45	0.3191
IL-1α	pg/ml	CBA	N.D.[c]	0.54 ± 2.09	0.3343
IL-1β	pg/ml	CBA	N.D.[c]	0.58 ± 2.24	0.3343
IL-10	pg/ml	CBA	N.D.[c]	0.69 ± 2.69	0.3343
IL-12p70	pg/ml	CBA	N.D.[c]	0.35 ± 1.36	0.3343
CX3CL1	pg/ml	CBA	N.D.[c]	6.55 ± 25.38	0.3343
CXCL8	pg/ml	CBA	12.93 ± 11.52	16.24 ± 7.05	0.3413
MMP-1	ng/ml	ELISA	5.19 ± 3.15	4.30 ± 3.67	0.5129
CCL2	pg/ml	CBA	67.08 ± 43.78	72.29 ± 59.36	0.7842
αCOLII Ab[b]	U/ml	ELISA	51.75 ± 37.95	263.93 ± 577.87	0.2109

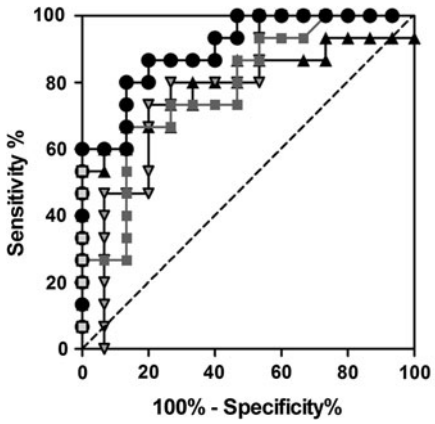
HD healthy donor, *RP* relapsing polychondritis, *sTREM-1* soluble triggering receptor expressed on myeloid cells-1, *ELISA* enzyme-linked immunosorbent assay, *IFN* interferon, *CBA* cytometric bead array, *ND* not detected, *CCL* chemokine (C–C motif) ligand, *VEGF* vascular endothelial growth factor *MMP* matrix metalloproteinase, *CXCL* chemokine (C–X–C motif) ligand, *hs-CRP* high-sensitivity C-reactive protein, *IL* interleukin, *TNF* tumor necrosis factor, *COMP* cartilage oligomeric matrix protein, *CX3CL* chemokine (C–X3–C motif) ligand, *αCOLII Ab* anti-type II collagen antibody

* By Welch's t test. p values of less than 0.05 are indicated in boldface

[a] The serum levels of IL-2, IL-5, GM-CSF, and CCL3 were below the detection limits in all cases

[b] The sample size of this item is different from that of the others due to the lack of some serum samples (HD: $n = 13$, RP: $n = 13$)

[c] For the statistical analyses, values of zero were substituted for the "N.D. (not detected)" entries



symbol	candidate markers	AUC	95%CI
●	sTREM-1	0.90	0.80 to 1.01
□	IFN-γ	0.77	0.59 to 0.94
▽	CCL4	0.79	0.62 to 0.96
■	VEGF	0.78	0.62 to 0.95
▲	MMP-3	0.80	0.63 to 0.97
---	reference line		

Fig. 1 Receiver operating characteristic (ROC) analysis of marker candidates of relapsing polychondritis (RP). We compared the sensitivity and specificity of soluble triggering receptors expressed on myeloid cells-1 (sTREM-1), interferon (IFN)-γ, chemokine (C–C motif) ligand 4 (CCL4), vascular endothelial growth factor (VEGF), and matrix metalloproteinase-3 (MMP-3) for discriminating RP patients from healthy donors (HDs) using ROC analysis. Closer proximity of the ROC curve to the upper left corner indicates higher sensitivity and specificity of the marker

IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70; interferon (IFN)-γ; tumor necrosis factor (TNF); chemokine (C–C motif) ligand (CCL) 2, CCL3, CCL4, CCL5; chemokine (C–X–C motif) ligand 8 (CXCL8), CXCL10; chemokine (C–X3–C motif) ligand 1 (CX3CL1); granulocyte–macrophage colony-stimulating factor (GM-CSF); and vascular endothelial growth factor (VEGF) were measured using a cytometric bead array (CBA; BD Biosciences, San Jose, CA, USA). All assays were conducted according to the respective manufacturers' instructions.

Immunohistochemistry

Biopsy specimens from three patients with RP chondritis were subjected to immunohistochemical analysis. Formalin-fixed tissue sections were deparaffinized in xylene and rehydrated in graded alcohols and distilled water. Slides were processed for antigen retrieval by a standard microwave-heating technique and incubated with anti-TREM-1 antibody (Sigma), followed by detection with streptavidin–biotin-horseradish peroxidase (Dako Cytomation Japan, Tokyo, Japan). All sections were visualized using 3,3′-diaminobenzidine (DAB).

Statistical analysis

GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) was used to plot graphs and perform statistical analyses. Mean serum concentrations of biomarker candidates were compared between RP patients and HDs using Welch's *t* test (Table 2). Receiver operating characteristic (ROC) analysis was used to examine the sensitivity and specificity of the selected markers (Fig. 1). Serum concentrations of biomarker candidates in patients with active RP and patients with inactive RP were analyzed by Welch's *t* test (Table 3). To compare serum sTREM-1 levels between healthy donors and patients with some inflammatory diseases (Fig. 3), we employed the Kruskal–Wallis test followed by Dunn's post hoc test. In all analyses, statistical significance was set at $p < 0.05$.

Results

Serum biomarker candidates in RP patients

First, we measured the serum levels of 12 cytokines, 7 chemokines, 4 MMPs, VEGF, hs-CRP, sTREM-1, COMP, and anti-type II collagen antibody in RP patients and age- and sex-matched HDs (Table 1), and compared the results from these two groups (Table 2). Serum samples from RP patients showed significantly higher concentrations of five molecules (sTREM-1, IFN-γ, CCL4, VEGF, and MMP-3) than the samples from HDs (Table 2). The serum levels of several other molecules (including hs-CRP, COMP, and anti-type II collagen antibody) tended to be higher in RP patients than in HDs, though the differences were not statistically significant.

Then, using ROC analysis, we compared the performances of the above five molecules in distinguishing RP patients from HDs. As shown in Fig. 1, the ROC analysis demonstrated that sTREM-1 had the highest sensitivity and specificity of the five molecules (area under the ROC curve [AUC] = 0.90; 95 % confidence interval [CI] 0.80–1.01; $p = 0.0002$). A sTREM-1 cut-off value of 158 pg/ml had a sensitivity of 86.7 % with a specificity of 86.7 %.

Table 3 Serum concentrations of biomarker candidates in patients with active RP and patients with inactive RP

Biomarker candidates[a]	Units	Active RP ($n = 8$) Mean ± SD	Inactive RP ($n = 7$) Mean ± SD	p*
sTREM-1	pg/ml	353.39 ± 158.03	200.14 ± 95.11	**0.0403**
VEGF	pg/ml	339.19 ± 218.10	185.48 ± 106.88	0.1066
hs-CRP	ng/ml	0.48 ± 0.64	0.10 ± 0.08	0.1342
TNF	pg/ml	1.43 ± 2.65	N.D.[c]	0.1708
IL-6	pg/ml	2.38 ± 4.45	N.D.[c]	0.1752
IL-17A	pg/ml	0.05 ± 0.14	0.71 ± 1.14	0.2129
MMP-3	ng/ml	334.71 ± 400.33	138.44 ± 135.59	0.2254
MMP-1	ng/ml	5.35 ± 4.35	3.07 ± 2.51	0.2658
MMP-13	ng/ml	0.30 ± 0.11	0.26 ± 0.05	0.3469
IL-1α	pg/ml	1.01 ± 2.86	N.D.[c]	0.3506
IL-1β	pg/ml	1.09 ± 3.07	N.D.[c]	0.3506
IL-10	pg/ml	1.30 ± 3.68	N.D.[c]	0.3506
IL-12p70	pg/ml	0.66 ± 1.87	N.D.[c]	0.3506
CX3CL1	pg/ml	12.29 ± 34.75	N.D.[c]	0.3506
MMP-2	ng/ml	139.68 ± 25.79	125.38 ± 31.39	0.3589
COMP	ng/ml	30.26 ± 35.31	17.56 ± 10.53	0.3598
CXCL10	pg/ml	251.14 ± 110.78	204.78 ± 121.20	0.4563
IFN-γ	pg/ml	4.54 ± 7.29	6.93 ± 5.06	0.4703
CXCL8	pg/ml	17.31 ± 6.34	15.01 ± 8.11	0.5571
CCL2	pg/ml	80.59 ± 78.04	62.80 ± 30.33	0.5660
CCL4	pg/ml	141.68 ± 90.46	124.71 ± 33.26	0.6332
IL-4	pg/ml	0.83 ± 2.36	0.76 ± 2.02	0.9509
CCL5	ng/ml	37.87 ± 17.21	37.42 ± 15.05	0.9585
αCOLII Ab[b]	U/ml	382.34 ± 808.48	162.44 ± 311.65	0.5525

RP relapsing polychondritis, *sTREM-1* soluble triggering receptor expressed on myeloid cells-1, *VEGF* vascular endothelial growth factor *hs-CRP* high-sensitivity C-reactive protein, *TNF* tumor necrosis factor, *N.D.* not detected, *IL* interleukin, *MMP* matrix metalloproteinase, *CX3CL* chemokine (C–X3–C motif) ligand, *COMP* cartilage oligomeric matrix protein, *CXCL* chemokine (C–X–C motif) ligand, *IFN* interferon, *CCL* chemokine (C–C motif) ligand, *αCOLII Ab* anti-type II collagen antibody

* By Welch's t test. p values of less than 0.05 are indicated by boldface

[a] The serum levels of IL-2, IL-5, GM-CSF, and CCL3 were below the detection limits in all cases

[b] The sample size of this item is different from that of the others due to the lack of some serum samples (active RP: $n = 6$, inactive RP: $n = 7$)

[c] For the statistical analyses, values of zero were substituted for the "N.D. (not detected)" entries

Identification of serum markers of disease activity in RP

Next, to identify a serum marker that correlates with RP disease activity, we divided the 15 RP patients into two groups based on the extent of inflammation (see "Methods" for details) (Table 1): active RP ($n = 8$) and inactive RP ($n = 7$). We then compared serum levels of all tested molecules in the two RP groups. The results showed that only serum sTREM-1 level was significantly higher in active RP patients than in the inactive RP patients ($p = 0.0403$) (Table 3). Moreover, to investigate the association of serum sTREM-1 level with disease activity in RP, we examined the clinical course of one patient with active RP. As shown in Fig. 2, treatment with methotrexate (MTX) provided symptomatic improvement in this case; simultaneously, the patient's abnormally high sTREM-1 level was reduced to almost the same level as healthy donor (720.5 pg/ml in Nov 2009 → 106.6 pg/ml in June 2011). Importantly, before the MTX treatment, the patient's CRP level was almost normal, even when the sTREM-1 level was abnormally high (CRP 0.41 mg/dl, sTREM-1 720.5 pg/ml).

Serum levels of sTREM-1 in patients with other immunological disorders

To investigate the disease specificity of sTREM-1, we measured the serum levels of this molecule in patients with other immunological disorders, including HTLV-1-associated

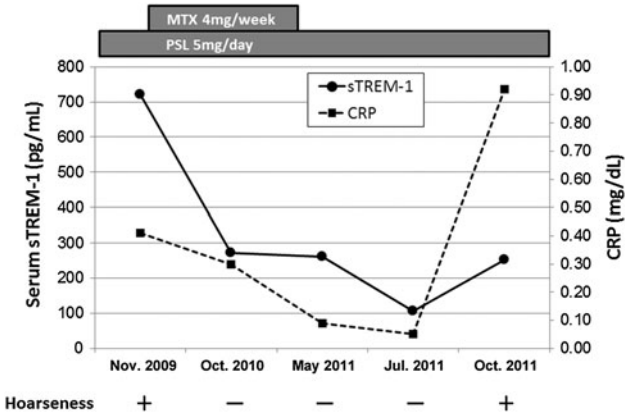


Fig. 2 Clinical course of a patient who was classified as having active RP at the time of enrollment, in 2009. The *line chart* shows the time courses of the serum sTREM-1 level (*closed circles*, *solid line*) and the CRP level (*closed squares*, *dashed line*) in an RP patient treated with prednisolone (PSL) and methotrexate (MTX). A plus sign (+) indicates the presence of hoarseness as a respiratory tract symptom, while a minus sign (−) indicates the absence of that symptom

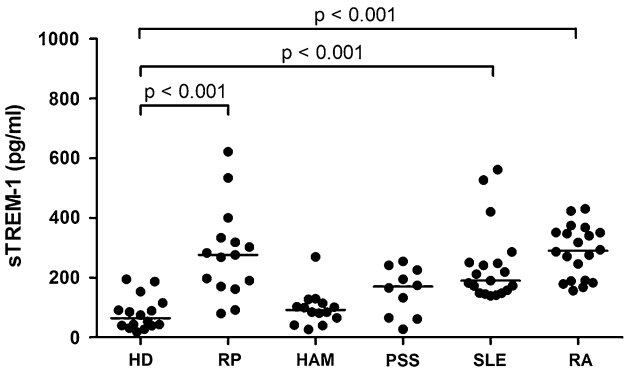


Fig. 3 Comparison of serum sTREM-1 levels between HDs and patients with other immunological disorders, including RP. Individual values are plotted, and the *bars* represent medians of the values. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's post hoc tests. *HAM* HTLV-1-associated myelopathy, *PSS* progressive systemic sclerosis, *SLE* systemic lupus erythematosus, and *RA* rheumatoid arthritis

myelopathy (HAM), progressive systemic sclerosis (PSS), systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA). Serum sTREM-1 levels were higher by a statistically significant amount in patients with RP and in patients with SLE or RA when compared to the levels in HDs (Fig. 3). This result indicates that elevation of the serum sTREM-1 level is not specific to RP.

TREM-1 expression in chondritis-affected areas of RP patients

Finally, we examined the expression of membrane-bound TREM-1 in chondritis-affected areas of RP patients. Immunohistochemistry demonstrated that TREM-1 was expressed on vascular endothelial cells in perichondral granulation foci but not on chondrocytes (Fig. 4). No positive cells were observed in a control sample (nonspecific inflammatory granulation tissue derived from a ruptured epidermal cyst) (Fig. 4).

Discussion

In this study, we identified serum sTREM-1 level as a novel biomarker for RP. We produced several results indicating the strength of this candidate marker: first, our results indicated that serum sTREM-1 level could discriminate RP patients from HDs more successfully than could other candidate biomarkers (Table 2; Fig. 1). Second, serum sTREM-1 level gave better discrimination between active RP patients and inactive RP patients than 27 other tested molecules, including hs-CRP, COMP, and anti-type II collagen antibody (Table 3). Third, the time course of serum sTREM-1 level was associated with the clinical course in an RP patient who was treated with prednisolone and MTX (Fig. 2). However, sTREM-1 showed some limitations in disease specificity, as its serum level was also elevated in patients with SLE or RA (Fig. 3). These results suggest that serum sTREM-1 level is suitable for use as a disease-activity marker for RP, but not as a diagnostic marker for the disease.

TREM-1, as the name suggests, has been shown to express on myeloid cells such as neutrophils and monocytes/macrophages [15]. Recently, it has been reported that TREM-1 is also expressed on endothelial cells (a type of non-myeloid cell) in liver tissue from lipopolysaccharide-treated mice [16]. In this study, our immunohistochemical analyses demonstrated that TREM-1 is expressed on human endothelial cells in chondritis-affected areas of RP patients (Fig. 4). The increase in sTREM-1 in the blood of RP patients might be due to its presence on the surfaces of endothelial cells in those inflammatory lesion sites. This hypothesis is supported by the finding that there was no difference in the expression level of TREM-1 on peripheral blood mononuclear cells between healthy donors and RP patients (data not shown). However, further investigations are needed to clarify the source of the increased sTREM-1.

It was previously reported that the expression of TREM-1 is induced by bacterial infection and that levels of circulating sTREM-1 are important as a diagnostic and prognostic marker of sepsis [17–19]. More recently, however, it has been reported that the serum sTREM-1 level is elevated in non-infectious chronic inflammatory diseases such as RA and inflammatory bowel diseases [20, 21]. Therefore, our finding that serum samples from patients with chronic inflammatory diseases (including RP, RA, and

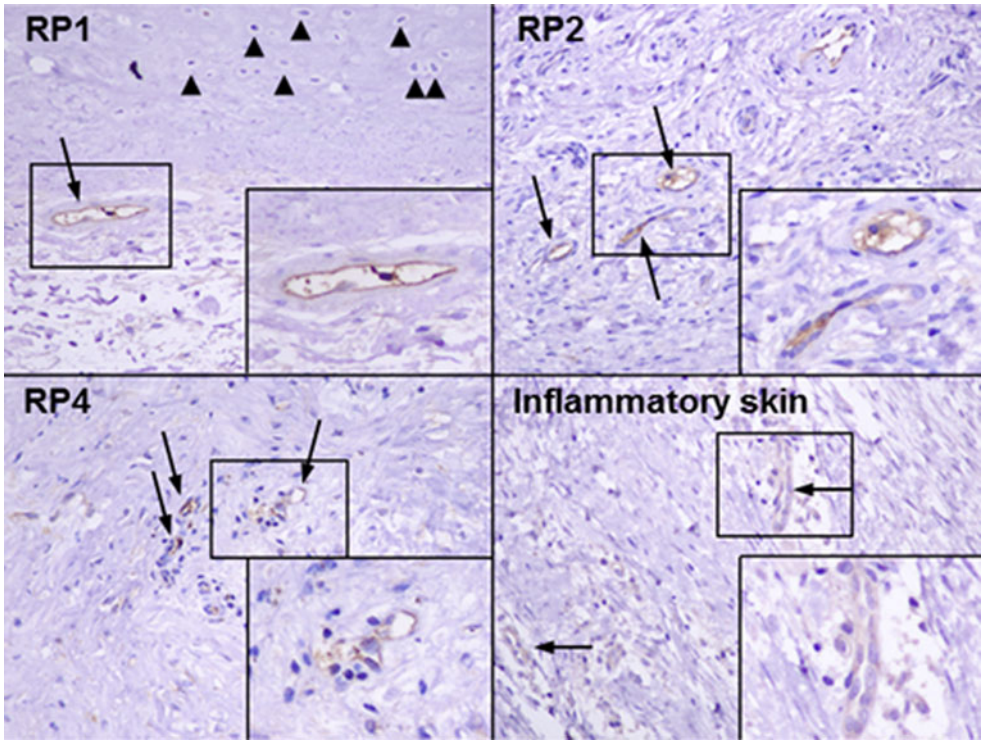


Fig. 4 Immunohistological staining showing the expression of TREM-1 in chondritis-affected areas. Inflammatory granulation tissue from a patient with a ruptured epidermal cyst was used as a negative control (*lower right panel*: inflammatory skin). TREM-1-positive cells were stained brown using 3,3′-diaminobenzidine (DAB) and are displayed at a higher magnification in the *lower right inset*. *Arrows* and *arrowheads* indicate vascular endothelial cells and chondrocytes, respectively

SLE) had significantly higher concentrations of sTREM-1 is consistent with previous reports. On the other hand, serum level of sTREM-1 in patients with HAM—a chronic inflammatory neurologic disease caused by human T cell leukemia virus-1—was not significantly higher than the level in HDs. This indicates that the serum level of sTREM-1 differs among patients with different chronic inflammatory diseases. Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a chronic inflammatory disease. Patients with AAV show elevated levels of serum sTREM-1 [22]. Intriguingly, as in RP, sTREM-1 levels in active AAV have been shown to be significantly higher than those for inactive AAV [22]. Thus, elevated levels of serum sTREM-1 have been observed in several chronic inflammatory diseases.

Such disorders with elevated sTREM-1 levels often overlap in the same patient. For example, 14 % of patients with RP have clinically evident vasculitis [23] and 35.5 % of patients have other collagen diseases, such as RA or SLE [24]. These examples imply the existence of common mechanisms in the pathogenesis of these disorders. In this regard, because TREM-1 works as an amplifier of inflammatory responses through the production of multiple pro-inflammatory cytokines and chemokines, TREM-1 may play an important role in the common pathomechanisms of these disorders [15, 21, 25, 26]. A previous study provided in vivo evidence that the blockade of TREM-1 can ameliorate collagen-induced arthritis in mice [27].

One of the molecules that has been reported as a disease-activity marker for RP is COMP [7]. This is a non-collagenous protein found in the matrix of cartilage. Lekpa et al. reported that serum COMP levels during the active phase were significantly higher than those seen during the inactive phase in the same patients. However, our results showed no significant differences in the serum levels of this molecule in active RP patients compared to inactive RP patients (Table 3). This discrepancy could be attributed to the different study designs employed, including differing disease conditions of the RP patients, sample sizes, and measurement methods.

To further characterize this molecule, we checked for correlations between serum levels of COMP and the other tested molecules. Interestingly, serum COMP levels in RP patients had a strong positive correlation only with serum MMP-3 levels ($rs = 0.7357$, $p = 0.0018$, by Spearman rank correlation test, data not shown). This suggests that serum levels of MMP-3 and COMP might reflect the degree of cartilage destruction in RP patients, since serum

MMP-3 level is considered a predictor of the degree of cartilage destruction in patients with early RA [28].

In conclusion, this study suggests that serum sTREM-1 level can serve as a more sensitive marker for disease activity in RP patients than other candidate molecules, such as CRP, COMP, and anti-type II collagen antibody.

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Conflict of interest None.

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Title page

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Title: A large-scale survey of patients with relapsing polychondritis in Japan

by

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Short running title: Airway involvement in relapsing polychondritis (42)

Abstract (247 words)

Relapsing polychondritis (RP) is a multisystem disorder characterized by recurrent inflammation and destruction of cartilage. The aim of this study is to assess the clinical characteristics of patients with RP in Japan, which remain unclear.

A survey was sent to 395 experienced clinicians who worked in Japanese major institutions. The questionnaire was designed to assess patients' profiles, clinical features, diagnosis, treatments and present complications. The response rate was 30.6% and 239 RP patient data were collected.

The average age of onset diagnosis was 52.7 years (range, 3-97) and the male-to-female ratio was 1.1:1. Clinical features of patients with RP in Japan were similar to previous studies. Airway and cardiac involvement, both of which were potentially serious complications of RP, were observed in 119 (49.8%) and 17 patients (7.1 %), respectively. Four patients (1.7%) had myelodysplasia. In addition to oral prednisolone (91.6%), patients received methotrexate (19.7%), cyclophosphamide (12.6%) and cyclosporine (8.4%) with clinical response rates of 64.0%, 66.7% and 73.7%, respectively.

42 patients (17.6%) required and underwent tracheotomy, including 12 patients (5.0%) who were treated with prednisolone only. 22 patients (9.2%) underwent stent placement and/or tracheotomy. The overall mortality rate was 9.0% (22 patients) and respiratory failure and pulmonary infection were the leading causes of death in patients with RP.

Airway involvement of RP was fundamentally progressive and required frequent clinical checks and appropriate intervention with administration of both prednisolone and immunosuppressant. Cardiac involvement of RP was less common in

Japan as compared with that in Western countries.

Key words: Airway involvement, Cartilage, Tracheal collapse, Steroid, Immunosuppressants.

Introduction

Relapsing polychondritis (RP) is an uncommon inflammatory disorder of unknown etiology that affects the cartilage of ear, nose, peripheral joints, and respiratory tract[1-4]. Other proteoglycan-rich tissues such as eye, inner ear, heart, blood vessels, and kidneys are also involved[1-4]. When the visceral is affected by inflammation, RP is a potentially lethal disease.

The epidemiological studies of this disease have been conducted in Caucasian population[4]. The incidence of RP in Rochester, Minnesota is estimated to be 3.5 cases per million populations per year[5]. It seems to occur with equal frequency in all racial groups, but there are very few data available on non-Caucasian populations. Several case series with a decade of RP patient data have been reported from South/North India[6, 7] and Singapore[8].

In 2012, RP Disease Activity Index (RPDAI), a preliminary score for assessing disease activity, was developed by worldwide specialists[9]. Nonetheless, even now, physicians treat patients with RP on the basis of largely empirical evidence because of the lack of large-scale survey and clinical guidelines for the management of patients.

Here, we conducted a survey of 239 patients with RP to outline the current epidemiology, clinical manifestations, management and long-term outcome of RP in Japan.

Subjects and Methods

A Multi-institutional study survey of Japanese major medical facilities was conducted from July to December 2009. All subjects who were sent the questionnaire were informed of the purpose of the study and the responses would be kept confidential. All the authors reviewed the questionnaire.

We performed preliminary survey of clinical experience to treat patients with RP in 1894 Japanese medical facilities on July 1st, 2009, using a surveillance definition as follows: larger bed sizes (+200 or university hospitals) and adequate functions for RP treatments (providing services with eye-throat-nose, respiratory, chest surgery, dermatology, neurology and rheumatology divisions). We also reviewed recent Japanese clinical reports and research articles of RP using web accessible medical literature databases made by US National Library of Medicine, Japan Medical Abstracts Society and Japan University hospital Medical Information Network, and sent the initial survey questionnaire to the authors. Then, a main survey was sent to the 395 physicians who have returned a mail to us that the physicians have been treating or treated at least one patient with RP on August 14th, 2009. The patient data of the survey questionnaire were collected anonymously. This survey was approved by the ethics committee of St. Marianna University School of Medicine.

The questionnaire consisted of 5 sections to assess patients' (a) profiles, (b) clinical features, (c) diagnosis, (d) treatments and (e) present complications. It was summarized in Table 1. We asked the physicians to give us the most current laboratory findings with respiratory function except the titers of anti-type II collagen antibody and pathological findings.

Results

The survey response rate was 30.6% (121 of 395 surveyed physicians) and 239 RP patient data were collected.

Patients' profiles

Patient characteristics in McAdam series[10)] and current survey were summarized in Table 2. The male-to-female ratio was 1.13:1 (127 males and 112 females). Uni-modal

age distribution of disease onset is indicated in Fig. 1. The average age at onset was 52.7 years with a range from 3 to 97 and the average disease duration was 5.3 years with a range from 1 to 33. The ratios of patients whose disease duration was shorter than 2 and 5 years were 25 and 65 % of whole patients, respectively. We suggested that the time to diagnosis was not so long because a large part of patients had relatively short duration of disease. Older people (more than 51 years old) tend to develop RP rather than younger people (0-20 years old).

Clinical features

Initial lesions and symptoms in patients with RP varied considerably. Auricular chondritis was shown in 137 patients (57.3%) and is the earliest and most frequent manifestation. 41 patients (17.2%) developed respiratory symptoms as an initial manifestation which included hoarseness, persistent cough, dyspnea, wheezing and inspiratory stridor caused by the inflammation of laryngeal, tracheal and bronchial cartilages.

Ocular symptoms (22 patients, 9.2%), arthritis (15 patients, 6.2%), inner ear disorder (9 patients, 3.8%), neurological symptoms (7 patients, 2.9%) and nasal chondritis (5 patients, 2.1%) were recognized in relatively small numbers of patients at the onset of disease.

The prevalence and severity of the disease symptoms increased during follow-up (Table 2).

Ninety-seven patients (40.6%), 47 patients (19.7%) and 119 patients (49.8%) showed tracheal lesion, laryngeal lesion and laryngotracheal involvement, respectively. Forty-nine patients (20.5%) suffered from upper airway collapse and 42 patients (17.6%) required tracheotomy. 22 patients (9.2%) underwent stent placement and 12

patients (5.0%) received nasal continuous positive airway pressure because of their tracheobronchomalacia.

Auricular and nasal chondritis were seen in 187 patients (78.2%) and 94 patients (39.3%), respectively. Saddle nose deformity after the nasal chondritis was observed in 76 patients (31.8%).

Otitis media complications with vestibular dysfunction were observed in 64 patients (26.8%). Prolonged inflammation in inner ear and vasculitis of internal auditory artery[2)] caused hearing loss (52 patients, 21.8%) and the vestibular dysfunction (39 patients, 16.3%) such as dizziness, ataxia, nausea and vomiting.

Joint, skin and eye involvement were observed in 92 (38.5%), 32 (13.4%), and 109 (45.6%) patients, respectively. The arthritis was mainly asymmetric, migratory and non-erosive.

Dermatologic manifestations included the purpura, papules, macules, vesicles, bullae, chronic dermatitis and nodules. Ocular symptoms included recurrent episcleritis, conjunctivitis, keratitis, uveitis, proptosis, periorbital edema, tarsitis and extra-ocular muscle palsy.

Neurologic and renal involvements were observed in 23 patients (9.6%) and 16 patients (6.7%), respectively. Cardiovascular involvement, including aortic insufficiency, myocarditis, pericarditis, paroxysmal atrial tachycardia, heart block and vasculitis, was observed in 17 patients (7.1%).

Laboratory findings

Most of patients with RP showed the elevation of erythrocyte sedimentation rate (ESR, 68.2%) and C-reactive protein (CRP, 86.2%). Urinalysis was usually normal. Although the data were not routinely available, matrix metalloprotease (MMP)-3 and antibody to

type II collagen were found in 20.1% and 13.8% of patients, respectively.

Conventional radiograph showed changes in larynx, trachea, surrounding soft tissues and bronchi. In two cases, respiratory tract involvement was assessed by laryngoscopy. Endobronchial ultrasonography revealed fragmentation and edema of tracheobronchial cartilage in two patients[11]. 3 dimensional-CT scan was performed in 61 patients (25.5%) and conventional CT was conducted in 30 patients (12.6%).

Biopsies were performed in 228 patients (95.4%) and 138 patients (60.5% of patients who underwent biopsy) were diagnosed with histological confirmation of RP.

Treatments

Main treatment for RP patients even with airway involvement remains medical management. In the medication history profile, non-steroidal anti-inflammatory drugs were administered alone for 8 patients (3.3%) who had mild auricular or nasal chondritis. 219 patients (91.6%) had received at least one course of prednisolone through oral administration (204 patients, 85.4% of all patients), intravenous infusion (17 patients, 7.1%) and pulse therapy (40 patients, 16.7%). Low daily dose of prednisolone was administered in the majority of patients. Minocycline hydrochloride was used in 8 patients with RP but its effect remained unclear.

Immunosuppressants which were used against the chronic progression of RP included methotrexate (MTX, n=47), cyclophosphamide (CPA, n=30), cyclosporin A (CYA, n=20) and azathioprine (AZP, n=22). MTX, CPA, and CYA elicited considerable effects on clinical outcomes in 64.0%, 66.7%, and 73.7% of patients, respectively. MTX was added as an adjuvant treatment in refractory RP patients who required higher maintenance doses of prednisolone to reduce the overall steroid requirement. 3 patients were maintained with MTX alone. AZP was less effective than other agents and the rate

was estimated as fewer than 40%. Tacrolims was used in 3 patients and ameliorated manifestations in one patient.

Of those 47 patients with the combined therapy of steroid with MTX, 20 patients (42.6%) had some respiratory symptoms and did not require any surgical intervention (Fig. 2). In contrast, all 12 patients (5.0% of all patients) treated with prednisolone alone underwent tracheotomy. CPA, CYA and AZP treatment in conjunction with steroid administration also reduced the prevalence of airway involvement in patients with RP (54.5%, 50.0% and 57.0%, respectively, Fig. 2).

Discovery of the central role of tumor necrosis factor (TNF)-α and interleukin (IL)-6 in autoimmune/inflammatory diseases and subsequent development of anti-cytokine agents have quickly led to the clinical application of them in treatment of refractory RP.

In our survey, infliximab, an anti-TNF-α agent, treatment resulted in a response in 6 cases of 10 RP patients with airway involvement that had not responded to conventional immunosuppressants. Etanercept (anti-TNF-α) and tocilizumab (anti-IL-6) treatment also showed a sustained response in 1 case of 3 patients with refractory RP.

Prognosis

We summarized the prognostic outcome of patients of RP in our cohort in Fig. 3. Medication was discontinued without any manifestation in 11 patients (4.6%). All these patients exhibited auricular chondritis without respiratory involvement and 2 of the patients had scleritis.

One hundred and fifty-nine patients (66.5%) were well controlled and, in total, 71.1% of patients in our cohort responded to the treatments. 32 patients (13.4%) showed limited response and 9 patients (3.8%) suffered from progressive disease or relapse. 22

patients (9.0%) died and the causes of death were as follows; respiratory failure (8 patients), pulmonary infection (4), cardiovascular disease (2), cerebrovascular disease (2), suicide (1), myelodysplasia (1), leukemia (1) and unknown reason (2).

Discussion

We conducted a large-scale survey of patients with RP in Japan. Surveyed physicians dispersed widely on geographic location and a large part of survey responses were limited in patient number even in the main surveys. Considering the survey response rate and the number of collected patient data, the RP prevalence in Japan was estimated to be similar to that in the United States[5].

The disease severity and prognosis of RP largely depends on airway and cardiovascular involvement[10]. It has been reported that airway involvement were seen in approximately half of all RP patients during follow-up, while the manifestation were observed in only 20% of the patients at the onset of disease[12, 13]. 10-30% of patients with airway involvement were treated with tracheotomy and the leading cause of death was airway collapse and/or pulmonary infection[12-14]. These study results were similar to those in our study (Table 2, Fig. 3). Several studies reported a female predominance in RP patients with airway involvement (male-female ratio, 1:2.3-2.8)[12, 13] but the ratio in our study was approximately 1:1.

It was suggested that the detection of tracheal wall thickness in CT scan was remarkably effective to the diagnosis of airway involvement in patients with RP and dynamic expiratory CT scan was more useful to indicate the lesions than conventional CT scan[12, 15-18]. Despite of the advances in CT scanning techniques, bronchoscopy is essential for the diagnosis because it identify additional findings in approximately 25% of RP patients who received the CT scan[12]. Miyazawa et al. described the

endobronchial ultrasonography was useful to indicate fragmentation and edema of cartilage in patients with RP[11].

It has been reported that cardiac involvement were seen in 15-46% RP patients and the second cause of RP death[10, 19]. The male-to-female ratio was high (1:0.4) in RP patients with cardiac complications[20]. A retrospective chart review of 33 RP patients with cardiac surgery recommended that ultrafast chest computed tomography, magnetic resonance imaging or trans-esophageal echocardiography should be repeated every 6 months because subclinical development of cardiovascular involvement was occasionally observed in RP patients[20].

Certainly, several reports have described the latent phase of cardiovascular complications for a few years after the onset of RP in relatively young patients[21-24]. Several RP patients developed febrile vasculitis after RP onset with or without anti-neutrophil cytoplasmic antibody (ANCA)[25-30]. The activity of the vasculitis correlated well with severity of scleritis in patients with RP[2, 31, 32].

In our study, we found that cardiovascular involvement was less frequent in Japan (7.1 %) as compared with other reports[10, 19]. The reason for the low prevalence of cardiovascular disease remains unclear. Low prevalence of cardiac complications was reported in Japanese patients with rheumatoid arthritis as well[33]. We speculate that this is a public health issue of Japanese people regardless of the presence or absence of diseases.

No specific laboratory diagnostic test exists for RP and the diagnosis is made by clinical features and pathological finings of chondritis[28]. Typical pathological changes began with the loss of proteoglycans' basophilic staining of cartilage. Then lymphocytes, plasma cells and neutrophils infiltrated into perichondrial area,

degenerated and decreased the number of chondrocytes. Finally, the cartilage was replaced by fibrous tissue[34].

In this study, tissue biopsies were conducted in 95.4% of patients with RP and a definitive diagnosis was obtained in 60.5% of patients who underwent biopsy. To reach accurate diagnosis of RP, it was essential that physicians perform a deep biopsy to obtain the chondral tissue in the site with acute inflammation[28].

In laboratory experiments of biopsy specimen, immunoglobulin and C3 component of complement deposited to margin of cartilage and perichondrial vessel wall[34]. Antibody to type II collagen was detected in patients with RP from the disease onset and the titers were correlated with disease activity[35]. Hyper-activation of macrophage/monocytes in peripheral blood of RP patients was reported using cytokine profile analysis[36].

We found that serum level of soluble triggering receptor expressed on myeloid cells 1 (TREM1), an inflammatory receptor on macrophage/monocytes, was correlated with disease activity[37]. These data suggest that over-activation of immune system in the whole organism of RP patients converge on the chondritis of RP in a polyphyletic manner.

Several studies reported the possibility that combination therapy with prednisolone and immunosuppressants was effective for patients with RP[21, 38, 39], especially that with methotrexate[1, 2, 40, 41]. In agreement with the studies, our survey revealed high prevalence of airway involvement in patients with prednisolone monotherapy and relatively low prevalence of the involvement in patients with the combination therapy. We recommend use of the combination therapy using prednisolone and immunosuppressants in RP patients with airway involvement.

We found several case reports showed the effectiveness of anti-cytokine antibodies, such as infliximab[42], adalimumab[43], anakinra[44, 45] and abatacept[46]. We presume that the biological agents are applicable for patients with refractory RP based on the results of this survey. However, it is important to control infections of respiratory tracts before administrating such biological agents.

Endoscopic and surgical interventions are sometimes unavoidable for respiratory distress and such interventions with experienced clinicians were effective for the treatment of airway involvement in patients with RP[12, 47]. The progression of airway involvement occurs even under intensive medication and intervention in some patients with RP and a new modality is awaited for treating such patients. We are currently planning to conduct a prospective study using a patient conducted patient registry system which allows us to collect detailed status data of patients.

Conclusions

We described here patient profiles and major clinical features in patients with RP in Japan. Airway involvement of RP was fundamentally progressive and required frequent clinical checks and appropriate medications. Combination therapy with prednisolone and immunosuppressants may be beneficial for controlling airway involvement of RP than prednisolone monotherapy.

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Disclosure

All authors have no conflict of interest.

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Figure legends

Fig. 1 Age distribution of disease onset in patients with RP

The mean age at onset of disease was 52.7 years old with a range from 3 to 97 years old. Older people (more than 51 years old) tend to develop RP rather than younger people (0-20 years old).

Fig. 2 Effects of immunosuppressants to the airway involvement of RP patients

Prevalence rates of airway involvement in patients with RP were 100, 42.6, 50.0 and 57.0% in treatment with steroid only, steroid with MTX, CPA, CYA and AZP, respectively, with and/or after each treatment.

Fig. 3 Summary of prognostic outcome in patients with RP in this survey

Medication was discontinued without any manifestation in 11 patients (4.6%). 159 patients (66.5%) were well controlled and, in total, 71.1% of patients in our cohort responded to the treatments. 32 patients (13.4%) showed limited response and 9 patients (3.8%) suffered from progressive disease or relapse. 22 patients (9.0%) died.

Table 1 Summary of Japan RP Questionnaire

a. Patients' profile
Sex, onset age, follow-up years and diagnostic delay
b. Clinical features
Primary and follow-up
c. Laboratory findings
Laboratory tests, image analysis and histopathologic features
d. Treatment
Non-steroidal anti-inflammatory, prednisolone, immunosuppressants, antibiotics and surgical intervention
e. Prognosis and complications
Tracheal collapse, tracheotomy, vulvar surgery and death

Table 2 Characteristics of patients with RP in McAdam series[10] and current survey

	McAdam (n=159)		Current survey (n=239)	
Profile				
Male-female ratio	83:76		127:112	
Mean age			57 (range 6-104)	
Mean age of disease onset	44		53 (range 3-97)	
Disease duration (yr)			5.3 (range 1-33)	
Clinical features (%)				
	Onset	Follow-up	Onset	Follow-up
External ear	26	89	57	78
Internal ear	6.4	46	3.8	27
Nasal cartilage	13	72	2.1	39
Airway	14	56	17	50
laryngo				20
tracheobronchial				41
Eye	14	65	9.2	46
conjunctivitis				15
scleritis				26
uveitis				11
Arthritis	23	81	6.2	39
Skin		17		13
Cardiovascular		24		7.1

Neurological	2.9	9.6
Renal		6.7
Myelodysplasia		1.7

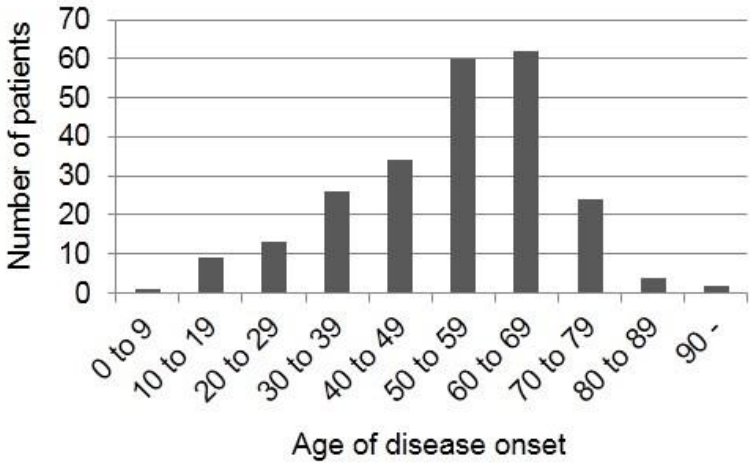
Number of patients
70
60
50
40
30
20
10
0
0 to 9
10 to 19
20 to 29
30 to 39
40 to 49
50 to 59
60 to 69
70 to 79
80 to 89
90 -
Age of disease onset

Fig. 1

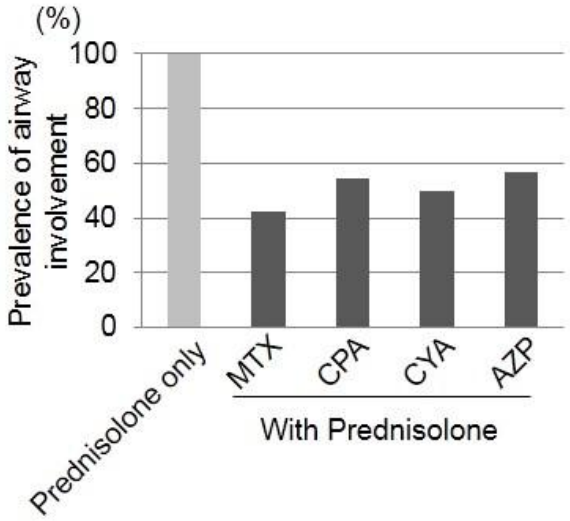
(%)
100
80
60
40
20
0
Prevalence of airway involvement
Prednisolone only
MTX
CPA
CYA
AZP
With Prednisolone

Fig. 2

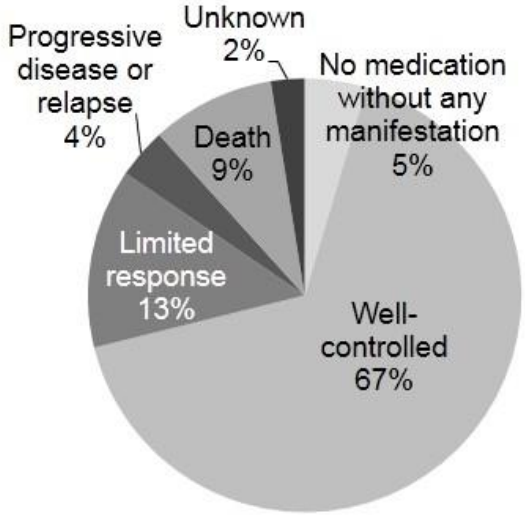
Unknown
2%
Progressive
disease or
relapse
4%
No medication
without any
manifestation
5%
Death
9%
Limited
response
13%
Well-
controlled
67%

Fig. 3