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-

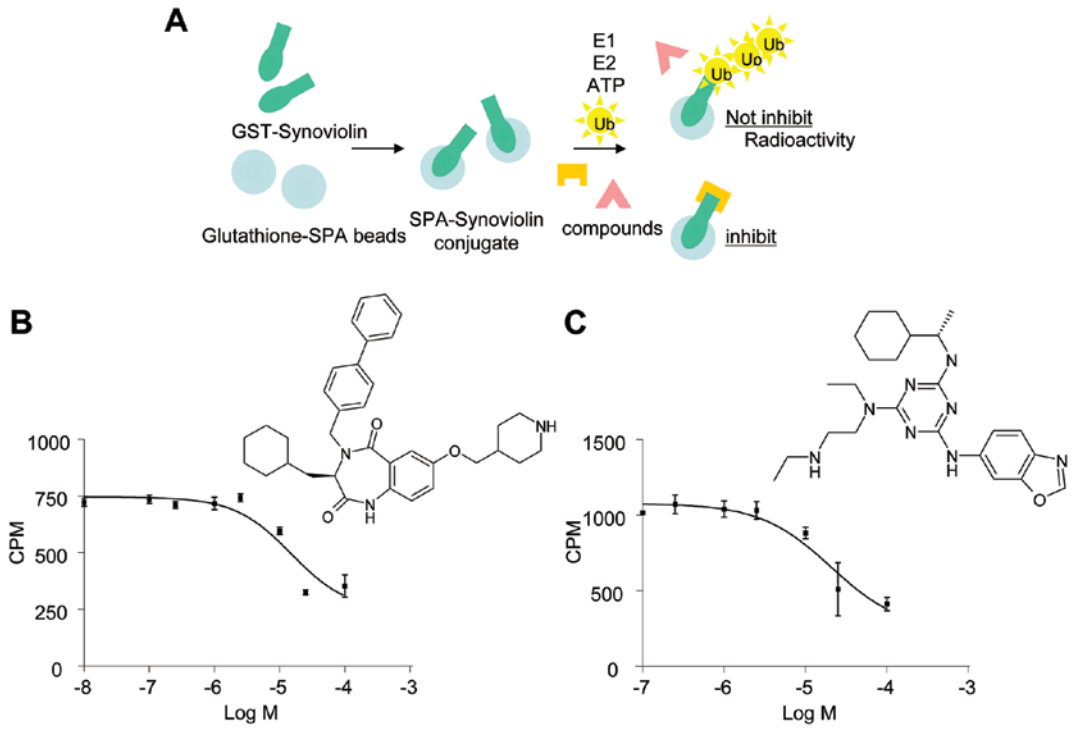

Figure 1. Screening for synoviolin inhibitors. (A) Scheme of high-throughput screening of synoviolin-induced ubiquitination assay. (B) Inhibition of synoviolin ^{33}P-polyubiquitination by LS-101 and chemical structure of LS-101. (C) Inhibition of synoviolin ^{33}P-polyubiquitination by LS-102 and chemical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

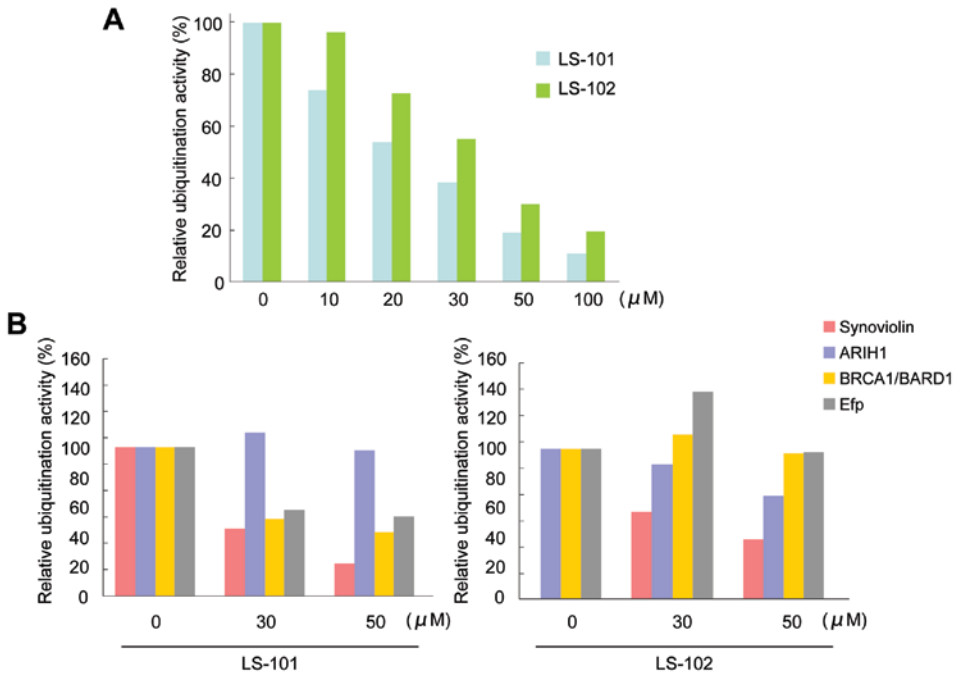

Figure 2. Effects of LS-101 and LS-102 on *in vitro* ubiquitination. (A) Both LS-101 and LS-102 inhibited the autoubiquitination of synoviolin in a dose-dependent manner. The IC_{50} of LS-101 was 20 μM and that of LS-102 was 35 μM. (B) Selectivity of LS-101 (left) and LS-102 (right) against other E3 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

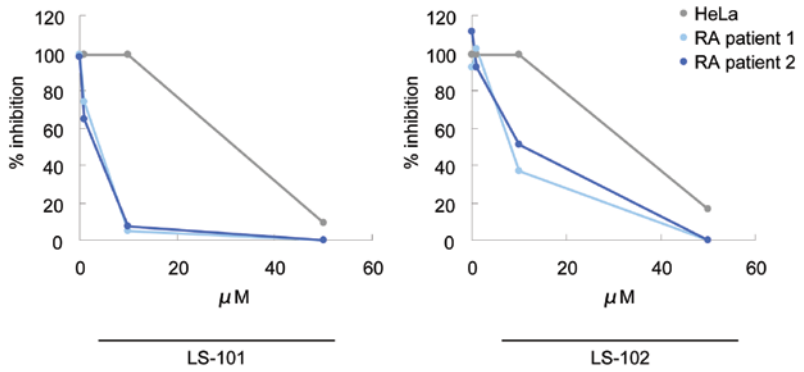

Figure 3. Effects of LS-101 and LS-102 on cell growth of RSCs. HeLa cells and RSCs derived from two RA patients were treated with synoviolin inhibitors for 12 h at the indicated concentrations. LS-101 and LS-102 repressed the proliferation of each RSC population tested. Data are expressed as the mean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

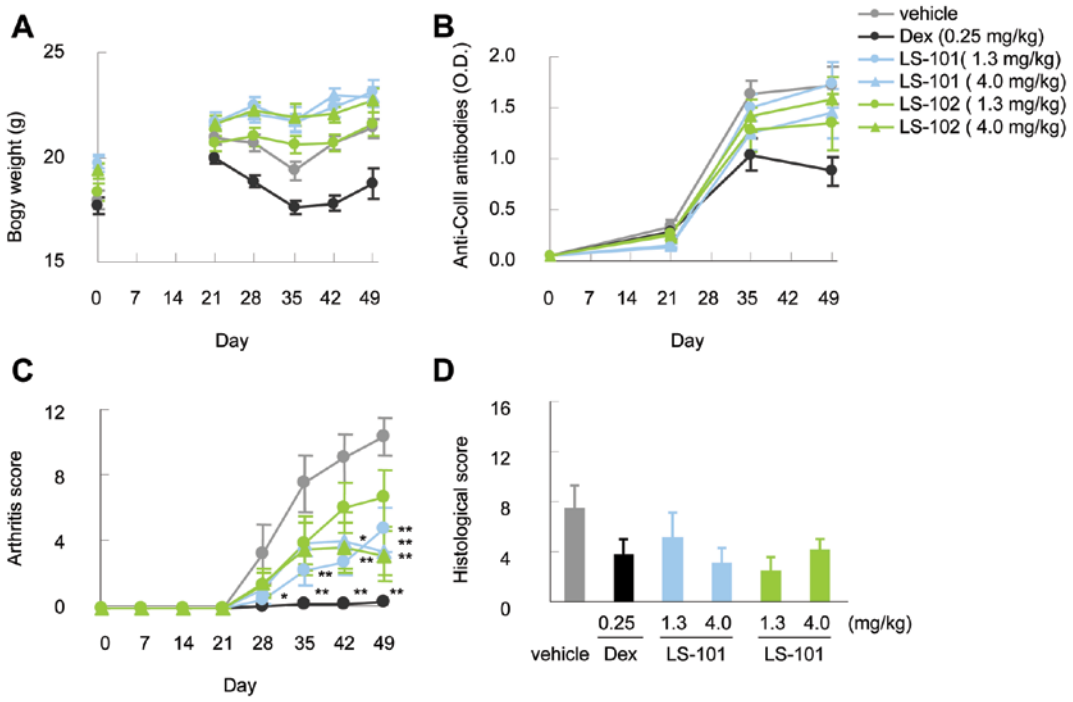

Figure 4. Effects of LS-101 and LS-102 in mouse CIA. DBA/1 mice immunized on day 0 and boosted on day 21 with type II collagen were treated with the vehicle alone, 0.25 mg/kg dexamethasone (Dex), or with 1.3, 4.0 mg/kg LS-101 or LS-102 from day 21 to 49. (A) Change in body weight. (B) The level of anti-type 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

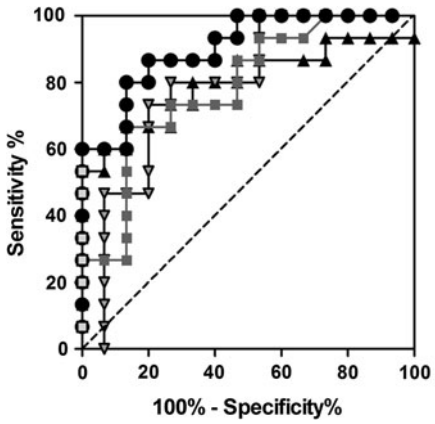
HD healthy donor, *RP* relapsing polychondritis, *sTREM-1* soluble triggering receptor expressed on myeloid cells-1, *ELISA* enzyme-linked immunosorbent assay, *IFN* interferon, *CBA* cytometric bead array, *ND* not detected, *CCL* chemokine (C–C motif) ligand, *VEGF* vascular endothelial growth factor *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

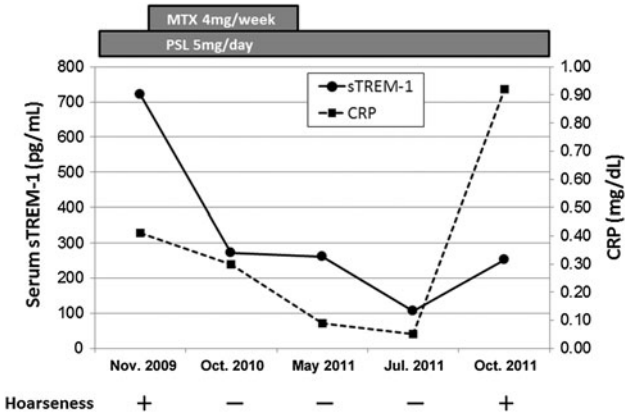


Fig. 2 Clinical course of a patient who was classified as having active RP at the time of enrollment, in 2009. The *line chart* shows the time courses of the serum sTREM-1 level (*closed circles*, *solid line*) and the CRP level (*closed squares*, *dashed line*) in an RP patient treated with prednisolone (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

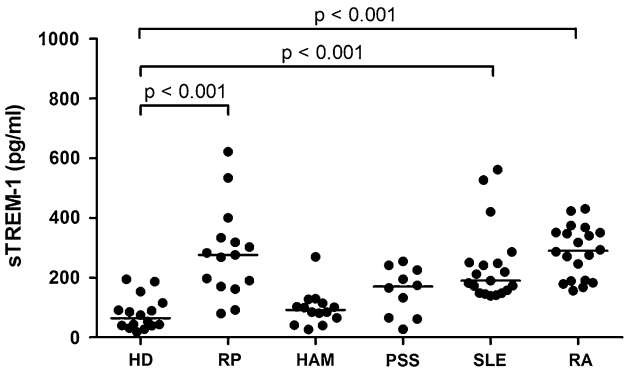


Fig. 3 Comparison of serum sTREM-1 levels between HDs and patients with other immunological disorders, including RP. Individual values are plotted, and the *bars* represent medians of the values. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's post hoc tests. *HAM* 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

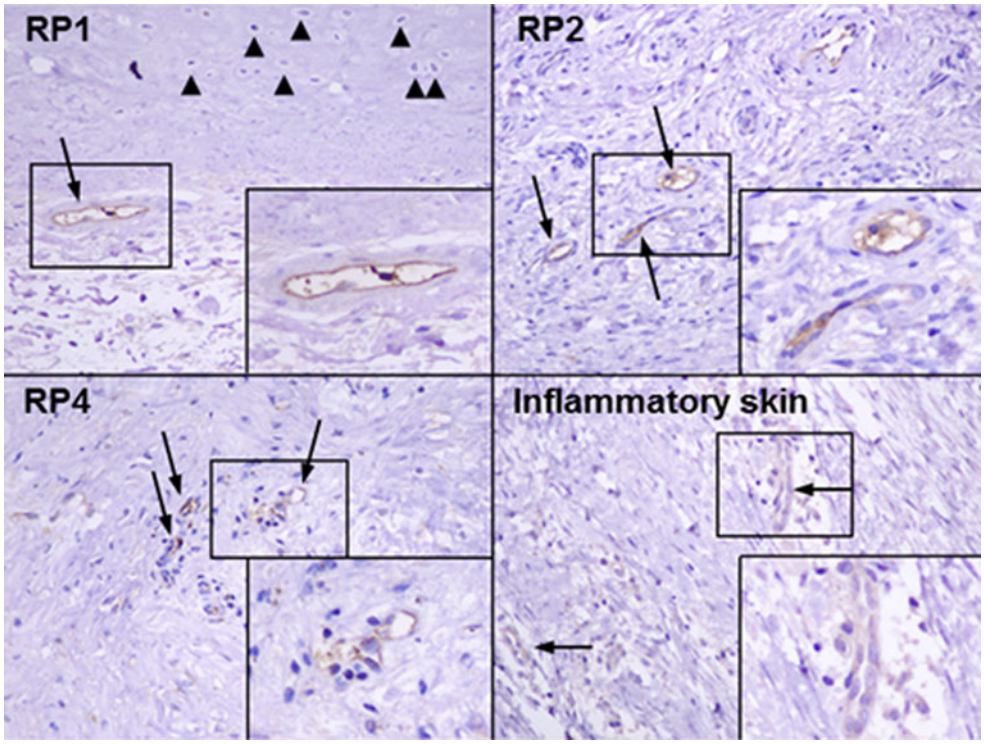


Fig. 4 Immunohistological staining showing the expression of TREM-1 in chondritis-affected areas. Inflammatory granulation tissue from a patient with a ruptured epidermal cyst was used as a negative control (*lower right panel*: inflammatory skin). TREM-1-positive cells were stained brown using 3,3′-diaminobenzidine (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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Nationwide Epidemiologic Study of Relapsing Polychondritis in Japan; results of 239 cases

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Background: The purpose of this study is the nationwide epidemiologic surveys of relapsing polychondritis (RP) in Japan.
Methods: The design is a retrospective survey. A questionnaire was sent throughout Japan to the medical doctors who have experienced the practice of RP patients. The requested data was consisted of patient profiles, clinical manifestations, examinations for diagnosis, therapeutic regimen and treatment response, and prognosis.
Results: The averaged age at the time of diagnosis was 52.7 years old, with a range from 3 to 97 years old. The male-to female ratio was almost equal. In laboratory findings, most of patients showed the elevation of C-reactive protein (84.5%) or erythrocyte sedimentation rate (68.5%), and in some cases, the elevations of matrix metalloprotease (MMP)-3 and antibody to type II collagen were found. However, some patients showed no elevation of these parameters with insidiously advancing fibrosis. Although the majority of clinical manifestations in Japanese RP patients were similar with those in Caucasians, airway involvement was observed in 49.8% of this cohort, and was strongly associated with the poor prognosis. For therapy, the prednisolone was used in 92% of the patients, and in some cases, additional immunomodulatory agents were used. The response rate to these agents was as follows; 64% to methotrexate, 66% to cyclophosphamide, 74% to cyclosporine, and 38% to azathioprine. While 71.1% of patients responded to therapy in some extent, 17.2% of patients showed no response, and 9.0 % of RP patients were dead. The cause of death was mainly associated with respiratory involvement.
Conclusions: The data of 239 patients with RP in Japan were collected. There was no gender-specific, geographic-specific distribution. The treatment response to azathioprine was lower than those to other immunomodulatory agents. The incidence of airway involvement was nearly 50 %, and was strongly associated with the poor prognosis.

INTRODUCTION

Relapsing polychondritis (RP) is an uncommon inflammatory disorder of unknown etiology characterized by an episodic and generally progressive course affecting predominantly the cartilage of the ear, nose, peripheral joints, and respiratory tract (1-4). Inflammation of other tissues such as the eyes, heart, blood vessels, inner ear, and kidneys may also be present (1-4). When the visceral is affected by inflammation, RP is a potentially lethal disease.

The epidemiological studies of this disease have been mainly done 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 cases of this disease have been reported from South India (6), North India (7), and Singapore (8). However, there have been no reports of nationwide surveillance of this disease both in Caucasians and non-Caucasians.

Furthermore, the choice of therapy for this disease is largely empiric, because there is little information about the natural history of RP and its response to therapy because its rarity and the lack of long-term follow up.

Here, we conducted the nationwide study about the epidemiology of RP in Japan, and present the clinical course, clinical manifestations, treatment, and the response to therapies of 239 patients with RP.

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METHODS

Questionnaire survey on relapsing polychondiritis.

Using the questionnaires, we asked about

beliefs and practices regarding RP management. In September 2009, we initially sent out the simple letter (totally 1894 letters) to ask whether they experienced RP patients. Next, to the hospitals with an experience of medical management of RP patients, a questionnaire was mailed to the 395 professionally active physicians who belong to the departments in charge of RP, at Japanese national, public and private universities, public or private hospital in Japan. The questionnaire was to be answered anonymously. This questionnaire survey was approved by the ethics committee of St. Marianna University School of Medicine.

The questionnaire was summarized in Table 1. The questionnaire consisted of 5 questions, of which questions 1 and 2 were related to patient profiles and clinical features, question 3 to examinations for diagnosis, questions 4 to treatments, and question 5 to prognosis and complications. Concerning epidemiology, question 1 was asked about the age at onset of the disease, sex, and duration of follow-up. Question 2 concerned the first and consequent symptoms in patients with RP. Question 3 concerned the diagnostic examinations; laboratory features, diagnostic imaging and histological analysis. Question 4 was related to the selection of treatments and also asked whether the selected treatments were useful or not. Question 5 was asked about the prognosis and complications.

A total of 121 questionnaires were returned (30.6 % response rate) and 239 (127 males, 112 females,) of the respondents had been diagnosed with RP by a professionally physician. These 239 patients were evaluated in the study.

RESULTS

Age and symptom at onset of the disease.

Of the 1894 hospitals to which primary letter were sent, 856 hospitals responded. Of those, 240 had experience and 616 had no experience of RP patients. Next, we sent secondary letter of questionnaire to 395 hospitals; of those, 121 reported one or more patients with RP.

The total number of RP patients reported in this survey was 239 (127 males, 112 females; male-to-female ratio was 1.13:1). The mean age at onset of disease was 52.7 years old, with a range from 3 to 97 years old. The distribution of age at onset of symptoms is shown in Figure 1. The onset of disease occurred in 4.2 % of patients at an age younger than 20 years old, in 31.0 % of patients between the ages of 21 and 50 years old, and in 63.6 % of patients at an age older than 50 years old. The most common age at onset of RP was 50 –60 years old.

Investigations for diagnosis.

Laboratory findings were non-specific. Most of patients with RP showed the elevation of erythrocyte sedimentation rate (ESR) in 163 patients and C-reactive protein (CRP) in 206 patients, consistent with acute or chronic inflammation. Urinalysis was usually normal. Although not routinely available, matrix metalloprotease (MMP)-3 and antibody to type collagen were found in 48 and 33 cases, respectively.

Conventional radiograph showed changes in the larynx, trachea and surrounding soft tissues as well as the bronchi of the lung. In two cases, respiratory tract involvement was assessed by laryngoscopy. Endobrochial ultrasonography revealed changes such as fragmentation and edema in the tracheobronchial cartilage in these two patients.

Biopsy from ear cartilage or other inflamed areas was performed in 228 cases (95.4%) of 239 patients in this survey, and 138 patients were diagnosed with histological confirmation.

CT scan was conducted in 91 cases out of 239 cases. Three dimensional-CT scan, which contributes to elucidate stenotic bronchial lesion, was performed in 61 cases, and conventional CT was in 30 cases.

Clinical features.

Of the 239 patients with RP, initial lesions and symptoms were as follows; auricular chondritis (137 patients: 57.3%), respiratory symptoms (41 patients: 17.2%) such as cough, hoarseness, difficulty of breathing, eye involvement (22 patients) such as scleritis, uveitis, arthritis (15 patients), inner air symptoms (9 patients) such as dizziness, difficulty in hearing, neurological symptoms (7 patients) such as headache and meningitis, nasal chondritis (5 patients), and so on.

The clinical feature observed in the whole course of disease was summarized in Table 2. 187 patients (78.2%) had auricular chondritis. The pain, red or violaceous dislocation, swelling and tenderness of the cartilagiou portion and the non-cartilagious lobe were present in almost all patients with auricular chondritis.

The nasal chondritis was seen in 94 patients (39.3%) of 239 RP patients. It presented with nasal pain, redness and swelling, nasal stuffiness, rhinorrhea or epistaxis. The saddle nose deformity by the destruction and collapse of the nasal bridge was observed in 76

patients (31.8%) of patients with nasal chondritis.

The vestibular dysfunction was observed in 64 patients (26.8%) in this survey. They showed the reduced hearing (52 patients: 21.8%) and the vestibular dysfunction (39 patients: 16.3%) with dizziness, ataxia, nausea and vomiting, which were caused by inflammation of the middle ear and audiovestibular structures and/or vasculitis of the internal auditory artery.

119 patients (49.8%) had laryngotracheal involvement [tracheal lesion: 97 patients (40.6%), laryngeal lesion: 47 patients (19.7%)]. The respiratory symptoms by the inflammation of the laryngeal, tracheal and bronchial cartilages included the hoarseness, persistent cough, dyspnea, wheezing and inspiratory stridor. 49 patients (20.5%) with respiratory symptoms showed the upper airway collapse caused by the damage to the laryngeal and epiglottal cartilage and required the trachestomy (42 patients: 17.6%), management with stents (22 patients: 9.2%) or nasal continuous positive airway pressure such as BiPAP (12 patients: 5.0%).

Non-erosive arthritis, skin involvement and eye involvement were observed in 92 (38.5%), 32 (13.4%), and 109 (45.6%) patients, respectively. Most of arthritis in this survey was asymmetric, migratory, seronegative and non-erosive joint symptom. Dermatologic manifestations included the purpura, papules, macules, vesicles, bullae, chronic dermatitis, or nodules on the limbs. The eye involvement included recurrent episcleritis, conjunctivitis, keratitis, or uveitis. Additional eye manifestations involved proptosis, periorbital edema, tarsitis and extraocular muscle palsy.

Neurologic and renal involvements in this survey 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%) of the 239 patients.

Treatments

Treatment has been symptomatic in this disease. Non-steroidal anti-inflammatory drugs (NSAIDs) alone were adequate for 8 patients with mild auricular or nasal chondritis.

More serious symptoms required the steroids (corticosteroid or prednisolone). Most patients (219 patients, 91.6%) had been, at least once, treated with steroids during a period of the disease (oral steroid: 204 cases, intravenous therapy: 17 cases, pulse therapy: 40 cases). Most patients require a low daily dose of prednisolone for maintenance. Minocycline hydrochloride was used in 8 patients with RP but obvious effects were not noted

Immunosuppressants used to control symptoms and progression of the disease, include azathioprine (AZP), methotrexate (MTX), cyclophosphamide (CPA), and cyclosporin A (CYA). As shown in Table 3, MTX, CPA, and CYA elicited considerable effects on RP progression. Effective rate of MTX, CPA, and CYA was 64.0%, 66.7%, and 73.7%, respectively. AZP, which effective rate was 37.6%, was less effective than these agents. Tacrolims was used in only 3 patients, and one of these patient showed responses.

In 47 refractory RP patients who require higher maintenance doses of prednisolone, MTX was administered as an adjuvant treatment. MTX was used with prednisolone to reduce the overall steroid requirement for disease control; however, 3 patients may eventually be maintained with MTX alone. Of 47 patients with the combined therapy of steroid with MTX, 20 patients (20/47 patients, 42.6%) had some respiratory symptoms. In contrast, all patients treated with steroids alone showed serious air involvements. CPA or AZP treatment in conjunction with steroid administration also showed a significant decrease of airway involvement (54.5% and 57.0%, respectively) while controlling symptoms (Figure 2).

Since advances in understanding of the pathological basis of inflammatory diseases have led to the development of biological therapies. The Discovery of the central role of tumor necrosis factor (TNF)-α and interleukin (IL)-6 in autoimmune diseases such as rheumatoid arthritis, and the subsequent introduction of the anti-TNF-α agents infliximab and etanercept, or the anti-IL-6 agent tocilizumab, have transformed the treatment of refractory RP.

In our survey, infliximab treatment resulted in a response in 6 cases of 10 RP patients with airway involvement that had not responded to conventional immunosuppressants (effective rate, 60.0 %). Etanercept or tocilizumab treatment also showed a sustained response in 1 case of 3 patients with refractory RP. Although biologics seemed to be potential therapeutic agents, very few cases which were reported in this survey were not sufficient to assess the efficacy and toxicity of these therapeutic agents in RP.

Prognosis

We also summarized the overall

prognosis of our cohort (Figure 3). 11 patients (4.6%) were cured. All these cured patients had auricular chondritis, 2 of them had scleritis, though they had no respiratory involvement. Furthermore, 159 patients (66.5%) were improved by the treatment. Thus in total, 71.1% of our cohort responded to the treatment in some extent. However, 32 patients (13.4%) showed no response to the treatment, 9 patients (3.8%) had become worse, and notably, 22 patients (9.0%) were died. The cause of death are as follows; respiratory failure (8 patients), pulmonary infection (4 patients), cardiovascular disease (2 patients), cerebrovascular disease (2 patients), and suicide (1 patient), MDS (1 patient), leukemia (1 patient), unknown (2 patients).

DISCUSSION

RP is characterized by recurrent and potentially severe inflammation of cartilaginous structure of the external ear, nose, peripheral joints and laryngo-tracheal organ. Cardiovascular tissue and eye were also involved because of its proteoglycan-rich structure.

We send our questionnaire to totally 395 experienced MD who belonged to university hospitals and national, public and larger private hospitals. Their specialties include departments of immunology, rheumatology, radiology, otopharyngolaryngology, internal medicine, dermatology, respiratory medicine and so on.

The diagnosis of RP was made depending upon the clinical features and pathological finings of chondritis, because no specific laboratory tests exist (9). Typical pathologic change was begun with the loss of proteoglycans' basophilic staining of cartilage. Then lymphocytes, plasma cells and neutrophils infiltrated perichondrial area, degenerated chondrocytes and decreased the number of them. Finally, the cartilage was replaced by fibrous tissue (10). Indeed, in this study histological examination of biopsied specimen was conducted in 228 cases out of 239 cases (95.4%). Typical pathological findings were present in 138 cases out of 228 cases who had histological examination. However, 90 cases were difficult to make a firm diagnosis of RP with the histological findings. This may be because wrong position of the biopsy site and/or missing the best timing of the biopsy such as biopsy after initiation of steroid administration.

The severity and prognosis of RP largely depends on laryngo-tracheal and/or cardiovascular involvement. It has been reported that laryngo-tracheal manifestations were seen in approximately 20%- 50% (11, 12) of all RP patients and one forth of patients with airway involvement were treated with tracheotomy (11). It is reported that the main cause of death is air way collapse and/or pulmonary infection, while air way symptoms were first manifestation in only 20% of RP patients with laryngo-tracheal involvement (13). These airway profiles were almost similar to the tendencies in this Japanese retrospective study.

In this survey, CT scan was conducted 38% of the patients in Japan. We rather recommend routine CT examination of the chest to find out lower respiratory tract involvement by expiratory phase scanning, and hopefully subclinical stenosis of bronchus. Behar JV et al. pointed out tracheal wall thickness in CT scan was very important to the diagnosis of RP (14-16). Dynamic expiratory CT scan is useful to evaluate patients air way but the range of sensitivity was wide (11, 17). Miyazawa T et al. described the endobronchial ultrasonography was useful in the diagnosis and treatment of RP (18).

There are a lot of case reports observing the efficacy of prednisone and immunosuppressant to the air way symptom (19). Recent studies reported the possibility that corticosteroid therapy and immunosuppressant combination may avoid the progression of air way involvement (20, 21).

In agreement with the above reports (19－21), our study revealed that all patients with airway involvement who had been treated with corticosteroid monotherapy resulted in tracheotomy, suggesting the insufficiency of corticosteroid monotherapy for the prevention of airway disease progression (Table 2). Therefore, we strongly recommend combination therapy of corticosteroid and immunosuppressant for those with airway involvement, even though the involvement is in their early phases.

Several studies reported the usefulness of interventions, such as balloon dilatation and stenting therapy. Our study and our own clinical experiences totally support this notion. Sarodia et al. mentioned successful uses of self-expanded metallic tracheobroncheal stents (22). Ernst A et al. reported the usefulness of the silicone stent.(11) They also described the progression of air way involvement even under their intervention and we recommend sufficient corticosteroid and immunosuppressant be administered to those patients.

It has been reported that cardiac involvement were seen in 15-46% RP patients (12, 23) and second cause of RP death. It was

more prominent in the male populations, while the ratios of female/male were even or high in whole RP patients. This complication includes aortic regurgitation and mitral regurgitation, myocarditis, pericarditis, heart block, ischemia, paroxysmal atrial tachycardia, and large artery aneurysm. In this study, we found that cardiovascular involvement was less frequent in Japan (7.1 %) as compared with other reports (15-46%). The reason for this discrepancy remains obscure. Further studies are necessary to confirm this tendency.

Dib C et al. reported the retrospective chart review of 33 patients with cardiac surgery (24). We agree their recommendation that because cardiac involvement can be *totally asymptomatic*, ultrafast chest computed tomography, magnetic resonance imaging, or transesophageal echocardiography important.

There were a few accounts of the study about the biological therapy on RP. First of all, Leroux et al. reported that Rituximab was *not* effective to RP in his retrospective study (25). We found several case reports showed the effectiveness of anti-cytokine antibodies, such as infliximab (26), adalimumab (27) and anakinra (28, 29). Based on this survey, we can not get any conclusion of the effectiveness of these biologics on RP at present. Some patients were obviously refractory to the biologics. Kraus et al. reported that MCP-1, MIP-1beta, and IL-8 were significantly higher in RP patients (AR2004; 50:3663-3667). Further efforts are need to elucidate cytokine involvement in the pathogenesis of RP.

In conclusion, we described here the initial Japanese large retrospective study of RP, and also mentioned two major complications of RP to understand the clinical aspect. We found that corticosteroid + immunosuppressant combination therapy is better than corticosteroid monotherapy for controlling airway involvement of RP. Further study is necessary to improve clinical outcome of this disease.

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Table 1. Summary of questionnaire used in this survey.

1. Patient profile
    - Age at onset of the disease
    - Age at diagnosis
    - Sex
    - Duration of follow-up
2. Clinical feature
    - First and/or consequent symptoms as following:
        - auricular chondritis
        - vestibular dysfunction
        - reduced hearing
        - arthritis
        - nasal chondritis
        - saddle nose
        - eye involvement
        - laryngotracheal involvement
        - cardiovascular involvement
        - skin involvement
        - nervous system involvement
        - kidney disease

3. Examinations for diagnosis
    Main laboratory features
    image analysis
    histopathologic features
4. Treatment (safety and efficacy)
    NSAID
    Steroid
    Immunosuppressive
    Antibiotics
    Surgical intervention
    Others
5. Prognosis and complications

Table 2. Frequency of clinical manifestations in 239 Japanese patients with RP

Clinical manifestations	frequency	(number of patients)
External ear	78.2 %	(187)
Internal ear	26.8 %	(64)
Nasal cartilage	39.3 %	(94)
Airway	49.8 %	(119)
Laryngo	17.2 %	(41)
tracheobronchial	33.9 %	(81)
Eye	45.6 %	(109)
conjunctivitis	14.6 %	(35)
scleritis	26.4 %	(63)
uveitis	10.5 %	(25)
Arthritis	38.5 %	(92)
Skin	11.4 %	(32)
Cardiovascular	7.1 %	(17)
Neurological	9.6 %	(23)
Renal	6.7 %	(16)
MDS	2.1%	(5)

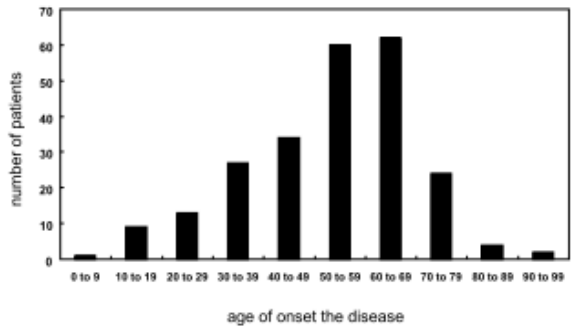

Figure1

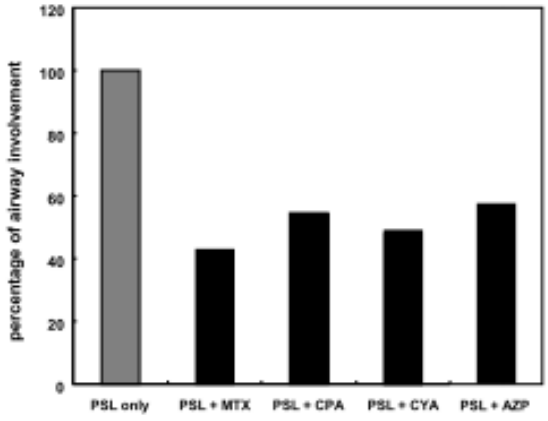

Figure2

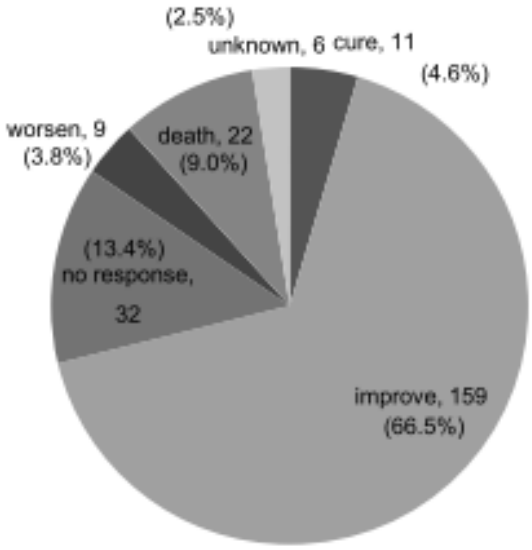

Figure3