inflammation in familial Mediterranean fever. Ann Rheum Dis 2010;69:677-82.
5 Morooka M, Kubota K, Murata Y *et al*. (18) F-FDG-PET/CT findings of granulocyte colony stimulating factor (G-CSF)-producing lung tumors. Ann Nucl Med 2008;22:635-9.
6 Mayer D, Bednarczyk EM. Interaction of colony-stimulating factors and fluorodeoxyglucose f(18) positron emission tomography. Ann Pharmacother 2002; 36:1796-9.
7 Kazama T, Swanston N, Podoloff DA, Macapinlac HA. Effect of colony-stimulating factor and conventional- or high-dose chemotherapy on FDG uptake in bone marrow. Eur J Nucl Med Mol Imaging 2005;32:1406-11.

Rheumatology 2011;50:1173-1175
doi:10.1093/rheumatology/ker128
Advance Access publication 30 March 2011

First report of anti-calcium-sensing receptor antibodies in a patient with Sjögren's syndrome and primary hypoparathyroidism

Sir, SS is a multisystem autoimmune disease that chiefly affects salivary and lacrimal gland function. The disease is characterized by lymphocytic infiltration of the exocrine glands, resulting in oral and ocular dryness, and the presence of anti-La and anti-Ro antibodies (Abs) [1]. Frequently, SS is associated with other autoimmune diseases including autoimmune hypothyroidism and Graves' disease [2-5]. In 1979, a patient with RA and dry eyes was reported to have hypoparathyroidism, but this was not proved to have an autoimmune origin [6]. Previous studies have demonstrated that hypoparathyroidism can have an autoimmune pathogenesis due to immune responses that either destroy the parathyroid or that give rise to Abs that aberrantly stimulate the calcium-sensing receptor (CaSR) on parathyroid gland chief cells [7-9]. The aim of the current study was to investigate the cause of hypoparathyroidism diagnosed in a patient with SS in order to determine whether an autoimmune aetiology was possible.

A 45-year-old female who satisfied 2002 criteria for SS was first found to be hypocalcaemic in 2001 with a corrected total serum calcium concentration of 2.04 mmol/l (normal range 2.15-2.65 mmol/l). The 25-hydroxyvitamin D level was <15 nmol/l (normal range 50-140 nmol/l) and calcium and vitamin D supplementation were given leading to normalization of 25-hydroxyvitamin D to 98 nmol/l. Serum magnesium, phosphate and alkaline phosphatase levels were normal. Hypocalcaemia fluctuating between 2.03 and 2.10 mmol/l persisted while 25-hydroxyvitamin D levels increased to 125 nmol/l. In 2009, serum PTH was undetectable at <10 ng/l (normal range 8-55 ng/l) paired with a low corrected serum calcium, a finding reproduced in a subsequent sample. The patient had a right-sided parotidectomy in 1995 revealing a lymphoepithelial lesion consistent with Mikulicz's syndrome. There had been no surgeries on her thyroid or parathyroid gland. Physical examination showed no features of other autoimmune conditions.

A diagnosis of primary hypoparathyroidism in this patient with SS was made when low serum calcium levels were found in the presence of a low level of PTH. The patient's normal renal function excluded the possibility of hypocalcaemia caused by renal disease and there was no evidence of intestinal dysfunction. Normal magnesium levels discounted this as a possible cause of insufficient PTH secretion. Serum calcium levels had remained normal for at least 4 years after parotidectomy increasing assurance that the parathyroid glands had been untouched. It is unlikely that hypoparathyroidism resulted from abnormally developed parathyroid glands or CaSR-activating mutations that reduce PTH secretion, as calcium levels were normal before 2000 and there was no suggestive family history. At least 30% of SS patients suffer from at least one additional autoimmune condition, most commonly hypothyroidism [2-5]. An autoimmune pathogenesis for this patient's hypoparathyroidism was therefore investigated by evaluating for the presence of anti-CaSR Abs [7-10].

Anti-CaSR Abs were measured in the patient's serum in a specific immunoprecipitation assay as previously described [10]. The patient was positive for anti-CaSR Abs with a CaSR Ab index of 21.6 (normal range, CaSR Ab index 0.15-1.72) (Fig. 1A). The effects of the patient's anti-CaSR Abs on CaSR function were determined using HEK293 cells expressing the CaSR (HEK293-CaSR cells). Cells were incubated with immunoglobulin G (IgG) before measurement of Ca^{2+}-induced, CaSR-mediated inositol-1-phosphate (IP1) accumulation and extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation [8]. The results indicated that the patient's IgG did not significantly affect the levels of either IP1 accumulation or ERK1/2 phosphorylation in HEK293-CaSR cells when responding to Ca^{2+} (Fig. 1B and C).

The main function of the CaSR is to regulate calcium balance by sensing changes in serum calcium concentration [11]. Anti-CaSR Abs have been shown to activate the CaSR leading to low levels of PTH secretion in patients with isolated autoimmune hypoparathyroidism and in the context of autoimmune polyendocrine syndrome type 1 [8-10]. The anti-CaSR Abs detected in this patient's serum did not appear to activate the CaSR. This may reflect low Ab levels that fail to stimulate the receptor in the functional assays used or non-activating anti-CaSR Abs that cause damage through complement fixation [7].

Hypoparathyroidism has only been reported in one previous case of SS that may not have satisfied 2002 criteria and in which an autoimmune pathogenesis for hypoparathyroidism was not proved [1, 6]. Our report, therefore, details the first case in which anti-CaSR Abs have been detected in a patient with SS and primary hypoparathyroidism. Ab-mediated salivary gland destruction has been demonstrated in an animal model of SS [12]. This patient's low PTH levels might have resulted from autoimmune parathyroid destruction that reflects the underlying pathogenic mechanism of SS [12]. Alternatively, an analogy can

Clinical Relevance of *MEFV* Gene Mutations in Japanese Patients with Unexplained Fever

To the Editor:

At the beginning of 2007, we investigated the frequencies of *MEFV* gene mutations in Japanese patients with unexplained fever or undifferentiated arthritis to determine their role in phenotypical features of familial Mediterranean fever (FMF)-related diseases. Patients were asked to complete a questionnaire concerning fever, recurrent typical attacks of FMF, including peritonitis, pleuritis, and arthritis, and transient inflammatory response. On the basis of the Tel-Hashomer criteria[1], we divided the study subjects into 3 groups, as follows: Group 1, typical FMF (presence of 1 or more major criteria independent of the presence of minor criteria); Group 2, probable FMF (absence of major criteria and 2 or more minor criteria); Group 3, unlikely (not belonging to either Group 1 or 2). Patients who had previously been diagnosed with typical FMF were not included. All patients were first enrolled as having unexplained fever, and finally diagnosed as FMF based on clinical evidence. We stress that the overall survey for the recent clinical manifestations, including the response to colchicine, was not complete in a few patients.

Up to January 2011, we had enrolled 142 Japanese patients with unexplained fever or undifferentiated arthritis in our genetic analysis. The subjects are 86 women and 56 men, with mean age of 38.2 ± 17.8 years. As shown in Table 1, 72 (50.7%) patients had single-nucleotide polymorphisms (SNP) of exon 2 of *MEFV* gene and 15 (10.6%) patients had SNP of exon 3 of *MEFV* gene. We identified 16 patients carrying a mutation of exon 10 (M694I): 3 were homozygotes, 11 were compound heterozygotes, and 2 were heterozygotes. All patients having the M694I mutation had typical episodes of serositis or monoarthritis in addition to periodic fever, and had been newly diagnosed as typical FMF. In contrast, the prevalence of FMF in patients with *MEFV* exon 1, 2, or 3 SNP was markedly lower (36.0%) than that in carriers of M694I. We compared the allele frequencies among typical or incomplete FMF patients and healthy subjects (35 women, 41 men, mean age 31.5 ± 8.0 yrs). The frequencies of M694I and E84K alleles were increased in patients with typical FMF, and frequencies of E148Q, P369S, and R408Q were increased in patients with probable FMF compared to healthy subjects ($p < 0.05$, Fisher exact test; Table 2).

We identified 12 patients carrying E84K mutation; clinical features of these patients are listed in Table 3. Among these 12, 4 had typical episodes of serositis or synovitis and periodic fever and could be diagnosed as typical FMF (Group 1). There was remittance of clinical symptoms with colchicine therapy in these patients with typical FMF, except for 1 patient (Patient 3) who remitted spontaneously. Another 2 patients carrying E84K mutation were considered to be "probable FMF" (Group 2); one patient remitted spontaneously, and colchicine was beneficial in the other patient. The remaining 3 patients carrying E84K mutation had atypical symptoms and did not fulfill a diagnosis of FMF (Group 3). In the last group, who had been diagnosed as having definite rheumatic diseases (Group 4), E84K mutation may have contributed the modification or sustained musculoskeletal symptoms to concomitant rheumatic diseases despite optimal treatment including steroid and immunosuppressants. We also analyzed the clinical features of the patients carrying the SNP of exon 1, 2, or 3 of the *MEFV* gene. Similarly, a subgroup of these patients were diagnosed as having typical or probable FMF (Table 4).

In our study, all 16 patients with M694I mutation were newly diagnosed as having typical FMF and showing the higher penetration of these mutations compared to that of exon 1 (E84K), exon 2 (L110P, E148Q, R202Q, G304R), or exon 3 (P369S, R408Q) mutations. Interestingly, we found 12 patients carrying a heterozygous E84K mutation who presented heterogeneous clinical phenotypes, in contrast to M694I carriers with typical FMF. Our findings indicated that a portion of the patients carrying E84K fulfilled the diagnostic criteria for typical FMF; however, more than half of these patients had atypical symptoms. We could not find any relevant clinical similarity in patients with E84K mutation, and the clinical phenotype of these E84K carriers might differ from the homogenous FMF phenotype. Our observations suggest that the *MEFV* gene mutations, which are attributed mainly to FMF, may also be responsible for additional clinical manifestations that do not meet the criteria of FMF as described[2,3]. A significant number of patients diagnosed as FMF have only a single mutation despite sequencing of the entire *MEFV* genome region or other autoinflammatory genes, and this has led to a reconsideration of the simple loss of function of the recessive model of FMF inheritance[4,5]. Recently, Chae, *et al*[6] demonstrated that gain-of-function pyrin mutations induce NOD-like receptor family, a pyrin domain containing 3 (NLRP3)-independent interleukin 1ß activation and autoinflammation. A plausible explanation might

Table 1. Genotypes of *MEFV* gene in patients with unexplained fever (n = 142).

MEFV Genotypes	n (%)	FMF Criteria: Typical (female 13, male 15)	FMF Criteria: Probable (female 11, male 6)
M694I/M694I	3 (2.1)	3	
M694I/normal	2 (1.4)	2	
M694I/E148Q	9 (6.3)	9	
M694I/E148Q/L110P	2 (1.4)	2	
P369S/normal	1 (0.7)		
P369S/R408Q	4 (2.8)		1
G304R/P369S/R408Q	1 (0.7)		
E148Q/P369S/R408Q	5 (3.5)		2
E148Q/E148Q/P369S/R408Q	2 (1.4)	1	
E148Q/R202Q/P369S/R408Q	1 (0.7)		1
E148Q/G304R/P369S/R408Q	1 (0.7)		1
E148Q/normal	22 (15.5)	3	3
R202Q/normal	4 (2.8)	1	
G304R/normal	2 (1.4)		
E148Q/E148Q	1 (0.7)		
E148Q/L110P	13 (9.2)	1	3
E148Q/R202Q	1 (0.7)	1	
E148Q/E148Q/L110P	4 (2.8)		2
E148Q/L110P/R202Q	1 (0.7)		1
E84K/normal	9 (6.3)	4	2
E84K/E148Q	1 (0.7)		
E84K/E148Q/L110P	2 (1.4)		
Normal	51 (35.9)	1	1
Total (%)	142	28 (19.7)	17 (12.0)

FMF: familial Mediterranean fever.

Table 2. Allele frequencies of *MEFV* gene mutations in Japanese patients with FMF and healthy subjects.

Alleles	Allele Frequencies (%): Typical FMF, n = 28	Probable FMF, n = 17	Healthy Subjects, n = 75
M694I	19 (33.9)*	0	
P369S	1 (1.8)	5 (14.7)*	6 (4.0)
R408Q	1 (1.8)	5 (14.7)*	5 (3.3)
L110P	3 (5.4)	6 (17.6)	13 (8.7)
E148Q	18 (32.1)	15 (44.1)*	35 (23.3)
R202Q	2 (3.6)	2 (5.9)	5 (3.3)
G304R	0	1 (2.9)	4 (2.7)
E84K	4 (7.1)*	2 (5.9)	2 (1.3)

* $p \leq 0.05$ compared to healthy subjects. FMF: familial Mediterranean fever.

Personal non-commercial use only. The Journal of Rheumatology Copyright © 2012. All rights reserved.

Table 3. Clinical manifestations of patients with E84K heterozygous mutation.

Case	Age, yrs	Sex, Age at Onset, yrs	Fever	Synovitis (arthritis)	Serositis	Other Clinical Symptoms	Response to Colchicine	Additional Mutation	FMF Criteria	Outcome	Concomitant Rheumatic Diseases
Group 1: typical FMF											
Case 1	14	M 14	+	—	Peritonitis	Myalgia	Good	—	Typical	Improved (colchicine)	—
Case 2	44	M 40	+	+	—	Myalgia	Good	—	Typical	Improved (colchicine)	—
Case 3	19	F 17	+	+	Peritonitis	—	Untreated	—	Typical	Remitted	—
Case 4	46	F 18	+	+	—	Osteomyelitis	Good	—	Typical	Improved (colchicine)	—
Group 2: probable FMF											
Case 5	17	F 14	–	+	Peritonitis	—	Good	—	Probable	Improved (colchicine)	—
Case 6	28	M 15	+	+	—	—	Untreated	—	Probable	Remitted	—
Group 3: undifferentiated											
Case 7	19	F 19	+ NT	—	—	—	Untreated	E148Q/–	—	Remitted	—
Case 8	9	M 8	+ NT	—	—	—	No response	E148Q/L110P	—	Sustained	—
Case 9	45	M 41	+ NT	+	—	Myalgia	Untreated	—	—	NA	—
Group 4: other rheumatic disease											
Case 10	59	F 58	+ NT	+	—	Myalgia	Untreated	—	—	Death (infection)	SLE + SSc
Case 11	6	M 6	+	—	—	Stomatitis, cervical lymphadenopathy	Untreated	—	—	Remitted	PFAPA
Case 12	50	F 41	+ NT	+	—	—	Untreated	E148Q/L110P	—	Sustained	AOSD

FMF: familial Mediterranean fever; AOSD: adult-onset Still's disease; NA: not available; NT: not typical; PFAPA: periodic fever with aphthous pharyngitis and adenitis; SLE: systemic lupus erythematosus; SSc: systemic sclerosis.

Table 4. Final diagnosis of patients with *MEFV* exon2 or exon3 single-nucleotide polymorphisms.

Patients	E148Q/–E148Q/ E148Q, n = 24	E148Q/L110P E148Q/E148Q/ L110P, n = 18	P369S/R408Q P369S/–, n = 6	P369S/R408Q/ E148Q, n = 9
Group 1 (typical FMF)	4	1		1
Group 2 (probable FMF)	3	6	1	4
Group 3 (undifferentiated)	13	8	4	4
Group 4 (other rheumatic diseases)	4	3	1	0
	Behçet disease (2) Sjögren syndrome Rheumatoid arthritis	Seronegative arthritis (2) Crohn disease	Behçet disease	

FMF: familial Mediterranean fever.

be that a subject having the *MEFV* single mutation carries a combination of polymorphisms that would favor more inflammation under the influence of a certain environmental factor and cross the threshold of manifesting an FMF phenotype[7]. These polymorphisms would be expected to belong to genes of the innate immune pathway[8]. Possible environmental factors are thought to be the patient's country of origin, with a geographically related, as yet unknown pathogenesis[9].

Our data showed a significant prevalence of FMF in Japanese patients with unexplained fever or undifferentiated arthritis; and we have to be more aware of the presence of a variant type of FMF or modification of other diseases by polymorphisms of the *MEFV* gene.

KIYOSHI MIGITA, MD, PhD, Clinical Research Center, Nagasaki Medical Center, Nagasaki; HIROAKI IDA, MD, PhD, Department of Rheumatology, Kurume University School of Medicine, Fukuoka; HIROYUKI MORIUCHI, MD, PhD, Department of Pediatrics, Nagasaki University Hospital, Nagasaki; KAZUNAGA AGEMATSU, MD, PhD, Department of Infection and Host Defense Graduate School of Medicine, Shinshu University, Nagano, Japan. Address correspondence to Dr. K. Migita, Clinical Research Center, NHO Nagasaki Medical Center, Kubara 2-1001-1, Omura 856-8652, Japan. E-mail: migita@nmc.hosp.go.jp

Personal non-commercial use only. The Journal of Rheumatology Copyright © 2012. All rights reserved.

Supported by a Grant-in-Aid for research on intractable diseases from the Ministry of Health, Labor and Welfare of Japan.

ACKNOWLEDGMENT
The authors acknowledge the participation of the following contributors: Seiyo Honda, MD, Department of Rheumatology, Kurume University School of Medicine; Tomohiro Koga, MD; Satoshi Yamasaki, MD; Atsushi Kawakami, Professor, Department of Rheumatology, Nagasaki University Hospital; Yuichiro Fujieda, MD, Department of Medicine II, Hokkaido University Graduate School of Medicine; Akira Morimoto, MD, Department of Pediatrics, Jichi Medical University School of Medicine; Yoshihisa Yamano, MD, Institute of Medical Science, St. Marianna University, School of Medicine; Iwadate Haruyo, MD, Department of Rheumatology Fukushima Medical University School of Medicine; Takeshi Mikawa, MD, Department of Pediatrics, Showa University School of Medicine; and Toshihiro Tono, MD, Department of Rheumatology and Infectious Diseases, Kitasato University School of Medicine.

REFERENCES

1. Livneh A, Langevitz P, Zemer D, Zaks N, Kees S, Lidar T, et al. Criteria for the diagnosis of familial Mediterranean fever. Arthritis Rheum 1997;40:1879-85.
2. Ben-Chetrit E, Peleg H, Aamar S, Heyman SN. The spectrum of MEFV clinical presentations — Is it familial Mediterranean fever only? Rheumatology 2009;48:1455-9.
3. Ryan JG, Masters SL, Booty MG, Habal N, Alexander JD, Barham BK, et al. Clinical features and functional significance of the P369S/R408Q variant in pyrin, the familial Mediterranean fever protein. Ann Rheum Dis 2010;69:1383-8.
4. Marek-Yagel D, Berkun Y, Padeh S, Abu A, Reznik-Wolf H, Livneh A, et al. Clinical disease among patients heterozygous for familial Mediterranean fever. Arthritis Rheum 2009;60:1862-6.
5. Booty MG, Chae JJ, Masters SL, Remmers EF, Barham B, Le JM, et al. Familial Mediterranean fever with a single MEFV mutation: Where is the second hit? Arthritis Rheum 2009;60:1851-61.
6. Chae JJ, Cho YH, Lee GS, Cheng J, Liu PP, Feigenbaum L, et al. Gain-of-function pyrin mutations induce NLRP3 protein-independent interleukin-1ß activation and severe autoinflammation in mice. Immunity 2011;34:755-68.
7. Ozen S. Changing concepts in familial Mediterranean fever: Is it possible to have an autosomal-recessive disease with only one mutation? Arthritis Rheum 2009;60:1575-7.
8. Ozen S, Berdeli A, Türel B, Kutlay S, Yalcinkaya F, Arici M, et al. Arg753Gln TLR-2 polymorphism in familial mediterranean fever: Linking the environment to the phenotype in a monogenic inflammatory disease. J Rheumatol 2006;33:2498-500.
9. Touitou I, Sarkisian T, Medlej-Hashim M, Tunca M, Livneh A, Cattan D, et al. Country as the primary risk factor for renal amyloidosis in familial Mediterranean fever. Arthritis Rheum 2007;56:1706-12.

J Rheumatol 2012;39;4; doi:10.3899/jrheum.110700

Personal non-commercial use only. The Journal of Rheumatology Copyright © 2012. All rights reserved.

Mod Rheumatol (2012) 22:45–51
DOI 10.1007/s10165-011-0461-4

ORIGINAL ARTICLE

Enhanced exon 2 skipping caused by c.910G>A variant and alternative splicing of *MEFV* genes in two independent cases of familial Mediterranean fever

Yumi Tone · Tomoko Toma · Akiko Toga · Yasuhisa Sakakibara · Taizo Wada · Masahiro Yabe · Hiromitu Kusafuka · Akihiro Yachie

Received: 4 April 2011 / Accepted: 15 April 2011 / Published online: 12 May 2011
© Japan College of Rheumatology 2011

Abstract Most reported cases of familial Mediterranean fever (FMF) involve missense mutations of *MEFV* concentrated within exon 10. We experienced two independent pedigrees of a unique variant in the *MEFV* gene that might cause excessive exon 2 skipping due to enhanced alternative splicing. In this study, we tried to elucidate the molecular mechanism of the *MEFV* variant as a cause of the FMF phenotype. Peripheral blood was obtained from volunteers and two patients with homozygous c.910G>A variant of the *MEFV* gene. *MEFV* messenger RNA (mRNA) expression patterns in mononuclear cells and granulocytes were compared using forward and reverse primers from exons 1 and 3, respectively. Expression profiles of pyrin were examined by transfecting wild-type and variant *MEFV* genes into HEK293T cells. Expression of normal-sized mRNA was extremely reduced in these patients, whereas that of aberrant short mRNA, deleting exon 2 (Δex2), was significantly increased. Immunohistochemical and immunoblotting analyses revealed a truncated immunoreactive pyrin protein in cells transfected with Δex2 cDNA. The *MEFV* gene c.910G>A variant results in accelerated aberrant splicing with abnormal protein size, presumably leading to anomalous pyrin function. This is the first report to show that an *MEFV* variant other than missense mutation is responsible for the FMF phenotype.

Keywords Familial Mediterranean fever · *MEFV* · Splice anomaly · Pyrin

Y. Tone · T. Toma · A. Toga · Y. Sakakibara · T. Wada · A. Yachie (✉)
Department of Pediatrics, School of Medicine, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, 13-1 Takaramachi, Kanazawa 920-8641, Japan
e-mail: yachie@staff.kanazawa-u.ac.jp

M. Yabe
Department of General Internal Medicine, Niigata City General Hospital, Niigata, Japan

H. Kusafuka
Department of General Internal Medicine, Nagoya Memorial Hospital, Nagoya, Japan

Introduction

Familial Mediterranean fever (FMF) is an autosomal recessive disease characterized by recurrent episodes of fever and serositis [1, 2]. Each episode of the attack continues for 1–4 days, and between attacks, symptoms can subside for several weeks to months. Left untreated, repeated episodes lead to AA amyloidosis and progression to renal failure. In some patients, unnecessary laparotomy is frequently performed due to recurrence of severe abdominal pain. Early diagnosis and appropriate therapeutic intervention is therefore mandatory to avoid these consequences. Although the vast majority of reported cases of FMF are from countries around the Mediterranean basin [3], as the name implies, patients from other part of the world are increasing in number [4, 5]. To date, more than 50 mutations in the FMF-associated gene (*MEFV*) have been identified in Japan since 1976 [6–8].

Mutations of the *MEFV* gene are concentrated within exon 10, and the major mutations—M694V, V726A, M694I, and M680I—comprise >74% of all reported mutations [3, 9, 10]. Notably, all of these mutations are missense, and no case has been reported with nonsense mutation or splice variants. In patients with FMF, pyrin dysfunction leads to aberrant molecular interactions

between these immunoregulatory proteins within inflammasome, resulting in the loss of regulatory function. The *MEFV* gene encodes pyrin, which is composed of five distinct domains. Among these domains, N-terminal PYRIN and C-terminal PRY-SPRY (B30.2) are thought to be responsible for major pyrin functions [11, 12]. PYRIN and PRY-SPRY associate with apoptosis speck protein (ASC) and caspase-1, respectively, and regulate the functions of inflammasome, leading to reduced levels of nuclear factor kappa-B (NF-κB) activation and interleukin-1β (IL-1β) production [13–16]. Among different *MEFV* mutations, the disease-related mutations within exon 10 have been the most thoroughly investigated to date. The functional interaction between the PRY-SPRY domain and caspase-1 appears to be inhibited in mutations involving M694 or M680, leading to excessive IL-1β production [17]. On the other hand, no mutation has been reported within the PYRIN domain, which has been shown to associate with ASC to control inflammation or to bind microtubules [17, 18].

In this report, we analyzed the molecular patterns of *MEFV* gene expression in two independent pedigrees of FMF patients who show a missense variant at the last nucleotide of exon 2. The patterns of mRNA expression and pyrin protein expression are shown for these cases.

Patients and methods

Patients

Patient 1 was a 61-year-old Japanese man who suffered from periodic episodes of fever and abdominal pain since the age of 43. The patient did not recall any episodes during his childhood. His abdominal pain was very severe and he had undergone repeated laparotomy under the diagnosis of appendicitis or cholelithiasis. No correct diagnosis was given for his suffering until his physician investigated the possibility of FMF. After we found the *MEFV* gene variant, he was started on colchicine. At the time of diagnosis, his serum C-reactive protein (CRP) was 6.3 mg/dl and white blood cell (WBC) count 3,200/μl. His symptoms resolved completely thereafter. No other person within his family showed similar symptoms.

Patient 2 was a 32-year-old Japanese woman with abdominal pain and lumbago. She was unrelated to patient 1, and her family history was unremarkable. Her lumbago developed at the age of 16. Since then, she experienced repeated episodes of severe abdominal pain of unknown origin and received laparotomy twice without any definitive diagnosis. Psychosomatic disease was the final diagnosis. She was treated with an antidepressant, without major improvement. She began having repeated febrile episodes 2 years prior to diagnosis, when FMF was suspected for the first time. At the time of diagnosis, her serum CRP was 0.1 mg/dl and WBC count 3,900/μl. An identical *MEFV* variant to that in patient 1 was found.

Gene analysis/sequence

Genomic DNA was isolated from peripheral leukocytes of the two patients and 125 healthy donors by standard procedures [19, 20]. We screened all *MEFV*-coding exons of DNA samples from patients and donors. Approval for the study was obtained from the Human Research Committee of Kanazawa University Graduate School of Medical Science, and informed consent from the two patients and all healthy donors was provided according to the Declaration of Helsinki.

MEFV mRNA/cDNA analysis

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll–Hypaque gradient centrifugation from patients and normal controls [21]. Granulocytes were recovered from the pellet of the gradient after lysis of erythrocytes. Total RNA of granulocytes and monocytes were isolated by Trizol reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA) was synthesized with the use of the oligo(dT)$_{20}$ primer and MMLV reverse transcriptase RNase H (ReverTra Ace, Toyobo Co., Ltd., Osaka, Japan) and amplified with GoTaq DNA polymerase using a forward primer located in exon 1 (5′-GTTCAAGCTGCAGAACACCAG-3′) and a reverse primer located in exon 3 (5′-AGTGTAAGCGCCAC CTGAAG-3′) of *MEFV*. The thermocycler conditions were as follows: one cycle of 5 min at 94°C followed by 40 cycles of 30 s at 95°C, 20 s at 60°C, and 30 s at 72°C, followed by final extension for 4 min at 72°C. The amplified products were separated by electrophoresis on a 1.5% agarose gel. The amplified fragments were detected by ethidium bromide staining of the agarose gel. Amplification of a fragment of the housekeeping gene β-actin was used as a positive control. Amount of amplified product was quantified by densitometry.

Activation of mononuclear cells by IFN-α or lipopolysaccharide (LPS)

PBMCs of patients and healthy controls were suspended in Roswell Park Memorial Institute (RPMI)-1640 containing 10% fetal calf serum (FCS) at 1×10^6/ml and stimulated with interferon alpha (IFN-α) (100 or 1,000 U/ml) or lipopolysaccharide (LPS) (10 or 100 ng/ml) for 8 h at 37°C in 5% carbon dioxide (CO_2). Total RNA was extracted from cultured cells, and *MEFV* mRNA expression was examined.

Plasmid construction and transfection for transient *MEFV* expression

The complete open reading frame of *MEFV* was amplified using specific primers (5′-CCGGAATTCGGCTAAGAC CCCTAGTGACCATCTG-3′ and 5′-ACGCGTCGACTG GGCATTCAGTCAGGCCCCTGACC-3′), and was subcloned into the pAcGFP1-C1 expression vector (Takara Bio, Otsu, Japan). To construct vectors carrying the G304R missense and the deleting exon 2 (Δex2) variants, the fragment of wild-type (WT) *MEFV* (exon 1–3) was replaced at the *Eco*RI and *Kpn*I sites with the variant sequences amplified from patient 1. All plasmid constructs were verified by sequencing. HEK293T cells were transfected with the vectors by lipofection using Lipofectamine™ 2000 (Invitrogen) and were harvested after 42-h culture at 37°C under 5% CO_2 [22].

Immunoblotting

Control HEK293T cells with mock plasmid (mock), WT *MEFV* gene, G304R single amino acid replacement (G304R) or pyrin with the exon 2 deletion (Δex2), or no transfectant were processed for immunoblotting with anti-pyrine antibody (R-13; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cell lysates were prepared with radio-immunoprecipitation assay (RIPA) lysis buffer (Santa Cruz) at 4×10^6/ml. Ku70 expression levels were determined simultaneously as internal controls with anti-Ku70 polyclonal antibody (M-19; Santa Cruz).

Immunofluorescence and immunohistochemistry

All cells were cultured in a Lab-Tek chamber slide (eight wells/slide, Nalge Nunc International, Naperville, IL, USA) for 48 h to obtain semiconfluent monolayers. After removing the medium chambers, slides were air-dried, fixed in acetone, and stored at −20°C until further analysis. Distribution of green fluorescent protein (GFP) was examined directly under a fluorescence microscope. Profiles of pyrin expression were examined by immunohistochemistry as described elsewhere using antipyrine antiserum [23].

Results

MEFV gene mutations

Figure 1 shows *MEFV* gene variants in our two cases. Homozygous c.910G>A variants were detected in DNA, which leads to the missense variants G304R. By screening 250 alleles from control donors, six alleles showed the identical variant. All of these control donors with *MEFV* variant were heterozygous carriers. According to National Center for Biotechnology Information Single Nucleotide Polymorphism (NCBI SNP) database, the frequency of c.910G>A variant is 1.4%, comparable with our analysis. No other mutation with amino acid substitution was found in either patient.

Increased Dex2 *MEFV* mRNA expression in patients of c.910G>A mutation

Reverse transcriptase polymerase chain reaction (RT-PCR) was performed using one set of primers spanning exon 2 of the *MEFV* gene (Fig. 2a). Although the short PCR products were seen in all six controls, the majority of products were 1,086-bp full-length products, with minimal short products. In marked contrast to the normal controls, most PCR products from the c.910G>A variant were short, with only trace levels of the full-length products (Fig. 2b). Calculation of the ratio of short to full-length products using densitometry data revealed significant differences between controls and patients (Fig. 2c). Both granulocytes and PBMCs from controls expressed five to ten times fewer short products than full-length products. In contrast, the ratio remained >2.5 in both granulocytes and PBMCs from the patients.

Short mRNA transcripts represent alternatively spliced *MEFV* mRNA

Full-length mRNA contained whole exon 2 with the G304R amino acid replacement (Fig. 3a). Direct gene

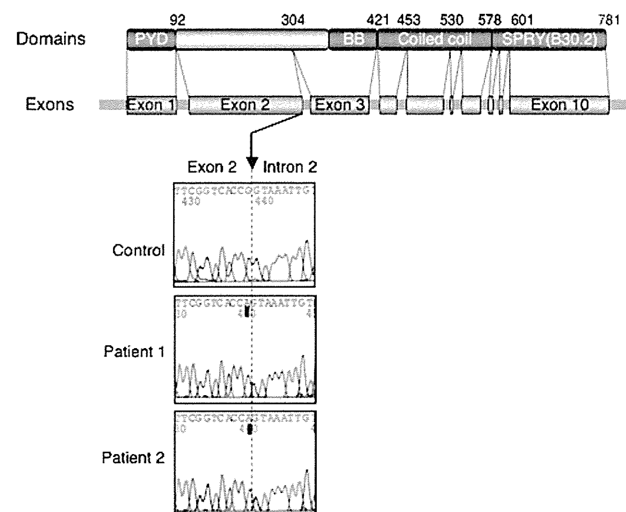

Fig. 1 *MEFV* gene structure and mutation analysis. Homozygous missense variant c.910G>A was found at the last nucleotide of exon 2 in patients 1 and 2. With normal splicing, this variant leads to a single amino acid replacement at the end of exon 2 (G304R)

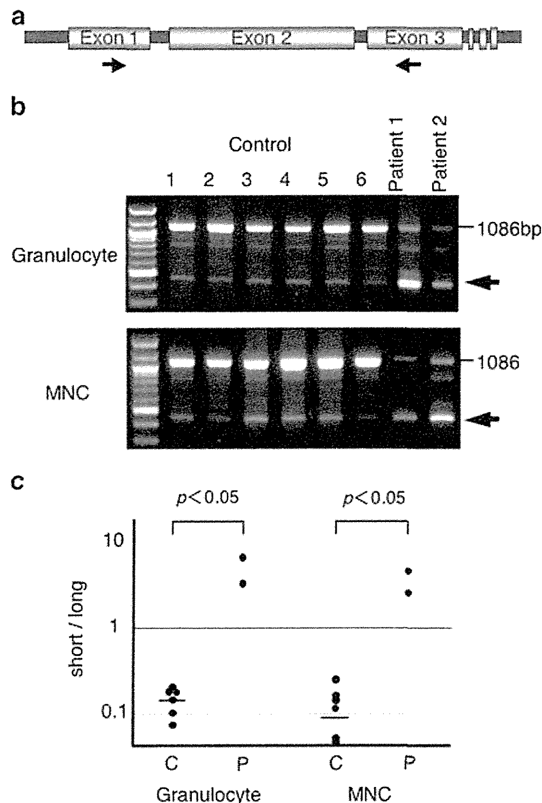

Fig. 2 Alternative splicing of the *MEFV* gene in c.910G>A patients. Both 5′ and 3′ primers were set within exons 1 and 3, respectively (**a**). In both granulocytes and peripheral blood mononuclear cells (PBMCs), full-length (1,086 bp) *MEFV* messenger RNA (mRNA) predominated in controls, and a small number of short product (*arrowheads*) was detectable (**b**). In contrast, in patients, the short product predominated both in granulocytes and PBMCs. Semiquantitative analysis of short to full-length ratio (**c**). Controls (*C*) showed a low short/full-length ratio, whereas patients (*P*) showed a significantly high short/full-length ratio, regardless the cell type

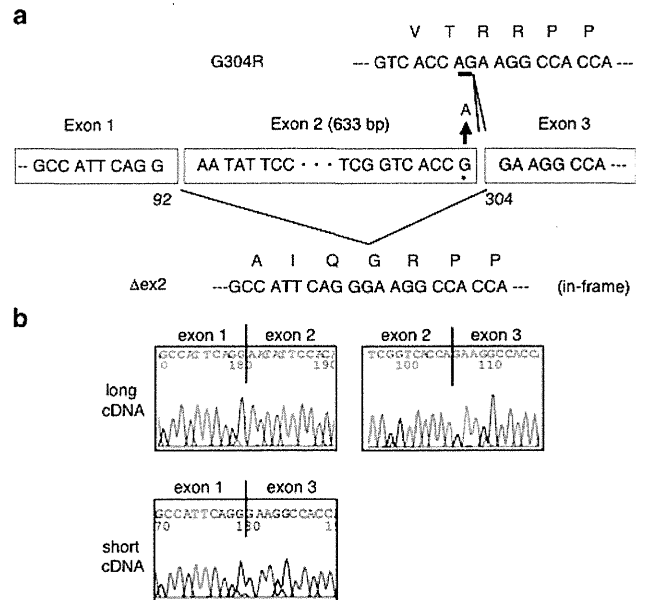

Fig. 3 Nucleotide sequences of alternatively spliced products. Organization of the nucleotide sequences is shown (**a**). Full-length (long) product showed single nucleotide replacement of c.910G>A, leading to the G304R amino acid replacement. Truncated product lacked whole exon 2, leading to an in-frame variant (Δex2). Raw sequencing data from the capillary sequencer (**b**)

sequencing of the short product indicated that the short mRNA transcript reflects the splice variant lacking the 633-bp exon 2 (Fig. 3b), suggesting that the mRNA is transcribed into the pyrin protein with the expected molecular weight of 90 kDa instead of 115 kDa.

MEFV mRNA induction by LPS and IFN-α

LPS or IFN-α stimulation has been reported to result in a significant increase in *MEFV* mRNA in PBMCs. LPS is also known to induce alternative splicing of *MEF*V in in vitro systems. We therefore examined whether enhanced *MEFV* mRNA transcription leads to altered splicing patterns in these patients. LPS and IFN-α treatment enhanced full-length *MEFV* mRNA transcripts in four controls (Fig. 4). Short mRNA transcripts were increased at the same time, but the predominance of full-length *MEFV* mRNA did not change after stimulation. Similar to controls, the short mRNA transcript level was increased after stimulation of patient PBMCs, whereas the full-length mRNA transcript level remained extremely low (patient 1) or paradoxically decreased (patient 2).

In vitro expression of the pyrin protein after *MEFV* gene transfection

To determine whether alternative splicing results in abnormal pyrin protein expression, we transfected normal and variant *MEFV* genes into HEK293T cells. In addition to normal WT cDNA, cDNA with the c.910G>A single nucleotide replacement (G304R), and exon-2-deleted gene (Δex2) were used (Fig. 5a). After transfection, expression profiles of the transfected genes were examined by immunofluorescence (Fig. 5b) and immunohistochemistry (Fig. 5c). GFP fluorescence was diffused within the cytoplasm of both WT and G304R transfectants. In contrast, Δex2 cells showed characteristic uneven clustering of GFP fluorescence. In all transfectants, GFP fluorescence remained within the cytoplasm, with no detection in the nuclei. Immunohistochemical examination of pyrin protein expression showed similar results. Namely, pyrin expression was observed diffusely throughout the cytoplasm of WT, G304R, and Δex2 transfectants but not within the nuclei. Immunoblotting analysis of cell lysates revealed that both WT and G304R transfectants express a pyrin protein of identical size ($M_W = 115$ kDa) (Fig. 5d), whereas the Δex2 transfectant expresses a truncated pyrin

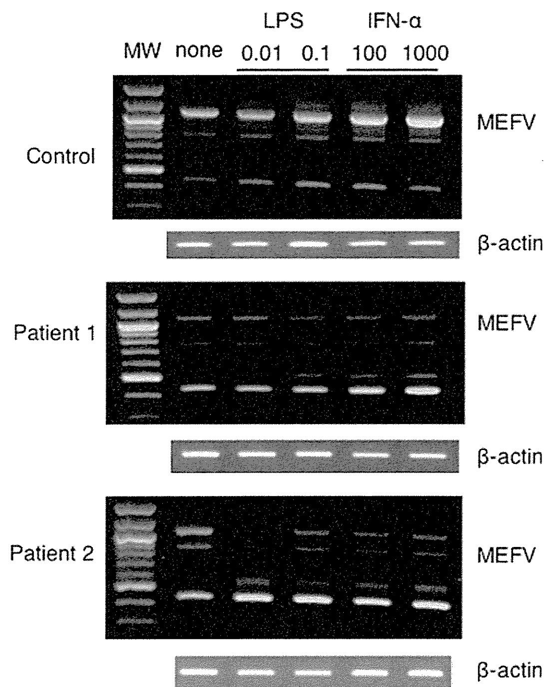

Fig. 4 Lipopolysaccharide (LPS)- and interferon alpha (IFN-α)-mediated regulation of *MEFV* messenger RNA (mRNA) levels. Peripheral blood mononuclear cells (PBMCs) were cultured alone or in the presence of LPS or IFN-α for 72 h. *MEFV* mRNA expression was compared among different samples. Expression of β-actin mRNA was used as the internal control

protein, as expected by the deletion of exon 2 ($M_W = 90$ kDa) (Fig. 5d). Pyrin was not detectable in nontransfected and mock-transfected cells. The levels of pyrin protein expression were similar among WT, G304R, and Δex2. Ku70 expression was also similar among the different transfectants (Fig. 5d).

Discussion

We report two unrelated cases of the same novel *MEFV* variant. The patients had homozygous c.910G>A variant, which results in excessive alternative splicing of exon 2 of the *MEFV* gene. To date, essentially all *MEFV* mutations of FMF patients are missense mutations [3, 4, 24, 25]. There has been no known report of nonsense mutation or mutation with exon skipping. Although the identical c.910G>A variant has been described as an SNP in the Internet Periodic Fevers (INFEVERS) Web site (rs 75977701), based on a personal communication, the associated phenotype of the reported patient was not described, and the causal role of the variant is unknown [26, 27]. Thus, this is the first report to propose the functional significance of this unique *MEFV* variant as a cause of variant FMF.

It was expected initially that the c.910G>A variant would result in the G304R missense variant with the full-length pyrin protein. To our surprise, most *MEFV* mRNA transcripts were short, and full-length mRNA transcript levels were significantly reduced in both patients. Because the c.910G>A variant is localized at the 3′ end of exon 2, alternative splicing may occur due to splicing errors. Direct sequencing of the short *MEFV* mRNA revealed that exon 2 was indeed deleted. Aberrant splicing of the *MEFV* gene has been reported previously [28, 29]. However, the anomalous splice products remained at low levels in these cases and did not indicate significant functional alterations of total pyrin activity. Similarly to these previous reports, the vast majority of *MEFV* mRNA observed in our control individuals was full length, and short mRNA with exon 2 skipping was seen only occasionally. Therefore, it is unlikely that individuals with heterozygous carrier of c.910G>A mutation show reduced level of full-length *MEFV* mRNA significant enough to have functional impairment of pyrin protein.

Centola et al. [30] previously showed that stimulation of peripheral blood monocytes with IFN-α or LPS resulted in augmented expression of *MEFV* mRNA in vitro, suggesting that an inflammatory environment may alter the expression profile of *MEFV* in vivo. Furthermore, Diaz et al. [29] reported that multiple alternative splicing events, including Δex2, is induced in LPS-stimulated synovial fibroblasts. These findings indicate that *MEFV* mRNA is vulnerable to alternative splicing and the events are induced relatively frequently upon exogenous stimulation, although the frequency of the anomalous splicing remains very low. To rule out the possibility that the enhanced alternative splicing of *MEFV* mRNA seen in our patients is due to sustained exposure to cytokines or endotoxins, we compared the in vitro effect of these agents on the levels of alternative splicing between the two patients and the controls. Stimulation of PBMCs with either IFN-α or LPS did not alter the patterns of *MEFV* mRNA expression in either controls or patients, strongly indicating that excessive levels of short *MEFV* mRNA observed in our patients is the direct result of the c.910G>A variant rather than in vivo augmentation of anomalous splicing, which was induced transiently upon exogenous stimuli. In this respect, the variant that results in significant reduction of full-length *MEFV* mRNA appears to be associated with dysfunction of the pyrin protein. Although it is possible that G304R amino acid substitution itself results in abnormal pyrin activity, its contribution to the total protein function is limited because of the low level of the full-length *MEFV* mRNA. Rather, predominance of the defective mRNA with anomalous splicing might explain the FMF-like clinical profiles seen in our patients.

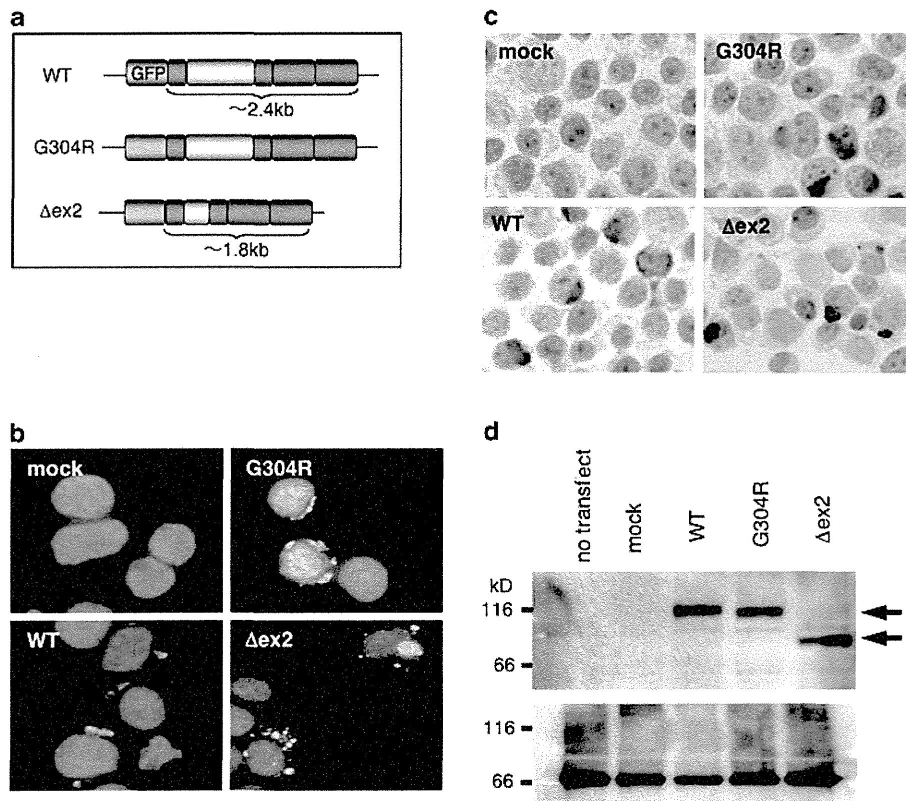

Fig. 5 *MEFV* gene transfection and pyrin expression profiles. HEK293T cells were transfected with plasmid containing the wild type (*WT*), G304R, or Δex2 *MEFV* gene (**a**). Plasmid containing only green fluorescent protein (GFP) was used as a control. Intracellular distribution of GFP was analyzed by immunofluorescence (**b**). Nuclei are stained blue with 4',6-diamidino-2-phenylindole (DAPI), and GFP fluorescence is localized within the cytoplasm in all transfected cells except mock-transfected cells. Pyrin protein expression was examined by immunohistochemistry. Positive staining is shown in *red* (**c**). The size of the pyrin products within the transfected cells was determined by immunoblotting (**d**). Although WT and G304R transfectants showed full-length protein (*upper gel, higher arrow*), the Δex2 transfectant showed only the truncated protein (*upper gel, lower arrow*). The *lower gel* shows the immunoblotting profiles of Ku70 used as a standard

Papin et al. [28] showed in vitro alternative splicing of exon 2 in the *MEFV* gene and an exon 2 deletion resulting in altered intracellular localization. Chae et al. [12] reported that the 15-aa bZIP basic domain and adjacent sequences, which are encoded by exons 2 and 3, interact with the p65 subunit of NF-κB and nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκB-α), respectively, and are important for nuclear translocation of the molecule. These findings indicate that exon 2 of *MEFV* is responsible for nuclear translocation of pyrin and exertion of its function within the nuclei. Notably, native pyrin expression was significantly different among cell types [29]. Namely, the pyrin protein was expressed predominantly within nuclei in dendritic cells, synovial fibroblasts, and neutrophils while being exclusively expressed within the cytoplasm of monocytes in irregularly dispersed patterns. These different patterns of intracellular distribution of native pyrin suggest that only certain types of cells are vulnerable to defective nuclear translocation and that functions of most other cell types remain intact, even when the nuclear translocation machinery is impaired.

Our own experiment using *MEFV* gene transfection failed to show altered intracellular pyrin distribution at least in HEK293T cells. Because HEK293T cells are of embryonic kidney origin, we need to examine the in vitro expression profiles of the transfected *MEFV* genes in other cell types to confirm whether different patterns are observed between Δex2 and control transfectants. Furthermore, native pyrin protein expression profiles should be compared among different cell types in patients when appropriate antipyrine antibody becomes available. The distinct and anomalous patterns of pyrin expression in different cell types in the patients, if present, may explain atypical clinical manifestation of this specific type of *MEFV* variant. Studies are underway to clarify these issues.

Acknowledgments We thank Ms. Harumi Matsukawa and Shizu Kouraba for their excellent technical assistance. This work was

supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan; and a grant from the Ministry of Health, Labour, and Welfare of Japan, Tokyo.

Conflict of interest None.

References

1. Samuels J, Aksentijevich I, Torosyan Y, Centola M, Deng Z, Sood R, et al. Familial Mediterranean fever at the millennium. Clinical spectrum, ancient mutations, and a survey of 100 American referrals to the National Institutes of Health. Medicine. 1998;77:268–97.
2. Onen F. Familial Mediterrenean fever. Rheumatol Int. 2006;26 :489–96.
3. Papadopoulos VP, Giaglis S, Mitroulis I, Ritis K. The population genetics of familial Mediterranean fever: a meta-analysis study. Ann Hum Genet. 2008;72:752–61.
4. Cornelius N, Duno M. Molecular evaluation of 458 patients referred with a clinical diagnosis of familial Mediterranean fever in Scandinavia. Rheumatol Int. 2010 (Epub ahead of print).
5. Halabe-Cherem J, Pére-Jiménez C, Nellen-Hummel H, Mercado-Atri M, Sigala-Rodríguez C, Castañón-González J. Familial Mediterranean fever in Mexico city. A 20-year follow up. Cir Cir. 2004;72:135–8.
6. Tomiyama N, Higashiuesato Y, Oda T, Baba E, Harada M, Azuma M, et al. MEFV mutation analysis of familial Mediterranean fever in Japan. Clin Exp Rheumatol. 2008;26:13–7.
7. Sugiura T, Kawaguchi Y, Fujikawa S, Hirano Y, Igarashi T, Kawamoto M, et al. Familial Mediterranean fever in three Japanese patients, and a comparison of the frequency of MEFV gene mutations in Japanese and Mediterranean populations. Mod Rheumatol. 2008;18:57–9.
8. Tsuchiya-Suzuki A, Yazaki M, Nakamura A, Yamazaki K, Agematsu K, Matsuda M, Ikeda S. Clinical and genetic features of familial Mediterranean fever in Japan. J Rheumatol. 2009;36: 1671–6.
9. Touitou I. The spectrum of familial Mediterranean fever (FMF) mutations. Eur J Hum Genet. 2001;9:473–83.
10. Brik R, Shinawi M, Kepten I, Berant M, Gershoni-Baruch R. Familial Mediterranean fever: clinical and genetic characterization in a mixed pediatric population of Jewish and Arab patients. Pediatr 1999;103(5). http://www.pediatrics.org/cgi/content/full.103/5/e70.
11. Ting JP, Kastner DL, Hoffman HM. CATERPILLERs, pyrin and hereditary immunological disorders. Nat Rev Immunol. 2006;6: 183–95.
12. Chae JJ, Wood G, Richard K, Jaffe H, Colburn NT, Masters SL, et al. The familial Mediterranean fever protein, pyrin, is cleaved by caspase-1 and activates NF-κB through its N-terminal fragment. Blood. 2008;112:1794–803.
13. Chae JJ, Wood G, Richard K, Park G, Smith BJ, Kastner DL. The B30.2 domain of pyrin, the familial Mediterranean fever protein, interacts directly with caspase-1 to modulate IL-1beta production. Proc Natl Acad Sci USA. 2006;103:9982–7.
14. Papin S, Cuenin S, Agostini L, Martinon F, Werner S, Beer HD, et al. The SPRY domain of Pyrin, mutated in familial Mediterranean fever patients, interacts with inflammasome components and inhibits proIL-1beta processing. Cell Death Differ. 2007;14: 1457–66.
15. Masumoto J, Taniguchi S, Sagara J. Pyrin N-terminal homology domain- and caspase recruitment damain-dependent oligomerization of ASC. Biochem Biophys Res Commun. 2001;280:652–5.
16. Richards N, Schaner P, Diaz A, Stuckey J, Shelden E, Wadhwa A, et al. Interaction between pyrin and the apptotic speck protein (ASC) modulates ASC-induced apoptosis. J Biol Chem. 2001;276:39320–9.
17. Mansfield E, Chae JJ, Komarow HD, Brotz TM, Frucht DM, Aksentijevich I, et al. The familial Mediterranean fever protein, pyrin, associates with microtubules and colocalizes with actin filaments. Blood. 2001;98:851–9.
18. Waite AL, Schaner P, Hu C, Richards N, Balci-Peynircioglu B, Hong A, et al. Pyrin and ASC co-localize to cellular sites that are rich in polymerizing action. Exp Biol Med. 2009;234:40–52.
19. Tone Y, Wada T, Shibata F, Toma T, Hashida Y, Kasahara Y, et al. Somatic revertant mosaicism in a patient with leukocyte adhesion deficiency type 1. Blood. 2007;109:1182–4.
20. Wada T, Yasui M, Toma T, Nakayama Y, Nishida M, Shimizu M, et al. Detection of T lymphocytes with a second-site mutation in skin lesions of atypical X-linked severe combined immunodeficiency mimicking Omenn syndrome. Blood. 2008;112:1872–5.
21. Mizuno K, Toma T, Tsukiji H, Okamoto H, Yamazaki H, Ohta K, et al. Selective expansion of $CD16^{high}CCR2^{-}$ subpopulation of circulating monocytes with preferential production of haem oxygenase (HO)-1 in response to acute inflammation. Clin Exp Immunol. 2005;142:461–70.
22. Subramanian S, Srienc F. Quantitative analysis of transient gene expression in mammalian cells using the green fluorescent protein. J Biotechnol. 1996;49:137–51.
23. Mizuno K, Yachie A, Nagaoki S, Wada H, Okada K, Kawachi M, et al. Oligoclonal expansion of circulating and tissue-infiltrating CD8+ T cells with killer/effector phenotypes in juvenile drematomyositis syndrome. Clin Exp Immunol. 2004;137:187–94.
24. Sabbagh AS, Ghasham M, Khalek RA, Greije L, Shammaa MR, Zaatari GS, et al. MEFV gene mutations spectrum among Lebanese patients referred for familial Mediterranean fever work-up: experience of a major tertiary care center. Mol Biol Rep. 2008; 35:447–51.
25. Ozdemir O, Sezgin I, Kurtulgan HK, Candan F, Koksal B, Sumer H, et al. Prevalence of known mutations in the MEFV gene in a population screening with high rate of carriers. Mol Biol Rep. 2010 (Epub ahead of print).
26. Touitou I, Lesage S, McDermott M, Cuisset L, Hoffman H, Dode C, et al. Infevers: an evolving mutation database for autoinflammatory syndromes. Hum Mutat. 2004;24:194–8.
27. Milhavet F, Cuisset L, Hoffman HM, Slim R, El-Shanti H, Aksentijevich I, et al. The infevers autoinflammatory mutation online registry: update with new genes and functions. Hum Mutat. 2008;29:803–8.
28. Papin S, Duquesnoy P, Cazeneuve C, Pantel J, Coppey-Moisan M, Dargemont C, et al. Alternative splicing at the MEFV locus involved in familial Mediterranean fever regulates translocation of the marenostrin/pyrin protein to the nucleus. Hum Mol Genet. 2000;9:3001–9.
29. Diaz A, Hu C, Kastner DL, Schaner P, Reginato AM, Richards N, et al. Lipopolysaccharide-induced expression of multiple alternatively spliced MEFV transcripts in human synovial fibroblasts: a prominent splice isoform lacks the C-terminal domain that is highly mutated in familial Mediterranean fever. Arthritis Rheum. 2004;50:3679–89.
30. Centola M, Wood G, Frucht DM, Galon J, Aringer M, Farrell C, et al. The gene for familial Mediterranean fever, MEFV, is expressed in early leukocyte development and is regulated in response to inflammatory mediators. Blood. 2000;95:3223–31.

Migita *et al. Arthritis Research & Therapy* 2012, **14**:R119
http://arthritis-research.com/content/14/3/R119

arthritis research&therapy

RESEARCH ARTICLE Open Access

Serum amyloid A triggers the mosodium urate -mediated mature interleukin-1β production from human synovial fibroblasts

Kiyoshi Migita[1,3*], Tomohiro Koga[4], Kenshi Satomura[1,2], Masahiro Izumi[1,2], Takafumi Torigoshi[1,2], Yumi Maeda[3], Yasumori Izumi[1], Yuka Jiuchi[3], Taiichiro Miyashita[1], Satoshi Yamasaki[4], Yoshihiro Aiba[3], Atsumasa Komori[3], Minoru Nakamura[3], Satoru Motokawa[1,2], Atsushi Kawakami[4], Tadashi Nakamura[5] and Hiromi Ishibashi[3]

Abstract

Background: Monosodium urate (MSU) has been shown to promote inflammasome activation and interleukin-1β (IL-1β) secretion in monocyte/macrophages, but the cellular pathway and nod-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome activation in synovial tissues, remain elusive. In this study, we investigated the effects of MSU on synovial fibroblasts to elucidate the process of MSU-mediated synovial inflammation.

Methods: Human synovial fibroblasts were stimulated with MSU in the presence or absence of serum amyloid A (SAA). The cellular supernatants were analyzed by immunoblotting using anti-IL-1β or anti-caspase-1 antibodies. IL-1β or NLRP3 mRNA expressions were analyzed by real-time PCR or reverse transcription-PCR (RT-PCR) method.

Results: Neither SAA nor MSU stimulation resulted in IL-1β or interleukin-1α (IL-1α) secretions and pro-IL-1β processing in synovial fibroblasts. However, in SAA-primed synovial fibroblasts, MSU stimulation resulted in the activation of caspase-1 and production of active IL-1β and IL-1α. The effect of SAA on IL-1β induction was impaired in cells by silencing NLRP3 using siRNA or treating with caspase-1 inhibitor. In addition, SAA induced the secretion of cathepsin B and NLRP3 mRNA expression in synovial fibroblasts.

Conclusions: Our data demonstrate that exposure of human synovial fibroblasts to SAA promotes MSU-mediated caspase-1 activation and IL-1β secretion in the absence of microbial stimulation. These findings provide insight into the molecular processes underlying the synovial inflammatory condition of gout.

Introduction

Gout is a paradigm for acute sterile inflammation that is triggered by interactions between monosodium urate (MSU) crystals and inflammatory cells in the joint connective tissues [1]. Interleukin-1β (IL-1β) has been identified as a pivotal cytokine in gout and MSU crystal-induced inflammation [2]. IL-1β is induced as an inactive pro-molecule by immune cells, such as macrophages and monocytes, and then cleaved into the active p17 form of IL-1 by caspase-1 [3,4]. Tschopp *et al.* demonstrated that MSU is capable of activating the NLRP3 inflammasome to process and secrete active IL-1β [5]. These findings suggest that macrophages can recognize MSU as danger-associated molecular patterns (DAMPs) in the damaged tissues and release proinflammatory IL-1β [6]. Upon activation, NLRP3 binds to the ASC, which in turn recruits procaspase-1 for activation. Activated caspase-1 cleaves pro-IL-1β to form the mature IL-1β [7].

In vitro experiments have shown that triggering of the inflammasome to process IL-1β is a multistep process. Lipopolysaccharide, which belongs to pathogen-associated molecular patterns (PAMPs), had been shown to induce IL-1β from human synovial macrophages [8]. In the absence of a first signal that induces pro-IL-1β, such as lipopolysaccharide, monocyte/macrophages do not spontaneously secrete mature IL-1β when stimulated with NLRP3 ligands [9,10]. Ii proposed that the first

* Correspondence: migita@nmc.hosp.go.jp
[1]Department of Rheumatology, Nagasaki Medical Center, Kubara 2-1001-1, Omura, Nagasaki 856-8562, Japan
Full list of author information is available at the end of the article

BioMed Central
© 2012 Migita et al.; licensee BioMed Central Ltd. This is an open access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

signal modulates the threshold of NLRP3 and the second signal activates NLRP3 inflammasome and causes subsequent caspase-1 activation and IL-1β processing [11,12]. Recent investigations demonstrated that IL-1β and IL-1 receptor are key players in MSU-mediated acute inflammation [13,14]. However, the steps that associate cellular activity with MSU crystals that induce inflammasome activation in gouty arthritis are not completely understood.

Serum amyloid A (SAA) is an acute-phase protein present in serum. SAA is also known to possess proinflammatory properties and to mediate inflammatory disease pathogenesis [15,16]. It has recently been demonstrated that β-amyloid fibrils in Alzheimer's disease signal through the NLRP3 inflammasome and drive caspase-1-dependent cleavage of IL-1β [17]. Furthermore, SAA has been shown to induce the expression of IL-1β and activate the NLRP3 inflammasome *via* a cathepsin B- and $P2X_7$-dependent manner [18]. In this study, we investigated the MSU-mediated NLRP3 activation process using synovial fibroblasts isolated from human synovium and adjuvant activity induced by SAA.

Materials and methods

Reagents

Recombinant human SAA was purchased from Peprotech (Rocky Hills, NJ, USA). According to the manufacturer, the endotoxin level of the product is 0.1 ng/mg protein. MSU crystals were purchased from Alexis (Lausen, Switzerland). Polyclonal anti-IL-1β, pro-IL-1β and anti-cleaved caspase-1 (D57A2) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-caspase-1 polyclonal antibodies (sc-622) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-NLRP3 antibodies were obtained from Abcam (Cambridge, UK). Anti-cathepsin B antibodies and caspase-1 inhibitor (z-YVAD-FMK) were obtained from Calbiochem (San Diego, CA, USA)

Preparation of synovial fibroblasts

Synovial tissues were obtained from patients with rheumatoid arthritis at the time of total joint replacement. Synovial fibroblasts were isolated from the synovial tissues by enzymatic digestion. The study was approved by the Ethics Committees Nagasaki Medical Center and informed consent was obtained from each of the individuals. Synovial fibroblasts were used from passages 4 through 6 during which time they are a homogeneous population of cells (<1% CD 45 positive).

Measurement of cytokine secretion and immunoblot analysis

Synovial fibrablasts (5×10^4) were seeded in 24-well plates containing RPMI1640 supplemented with 10% heat-inactivated FBS and stimulated with MSU for 24 hours. In some experiments, synovial fibrablasts were pre-treated with SAA for 12 hours before stimulation. Cell-free supernatants were collected by centrifugation at 400 g for five minutes and assayed for IL-1β or IL-1α with enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) without the steps for concentrations or precipitations. The same supernatants were also subjected to 12% SDS-PAGE, followed by immunoblotting with Abs for human IL-1β (dilution 1:400), caspase-1 (dilution 1:500), and cathepsin B (dilution 1:500) with an ECL Western blotting kit (Amersham, Little Chalfont, UK). Endotoxin was measured by chromogenic limulus test (Toxicolor LS-50M Kit, SEIKAGAKU CORPORATION, Tokyo, Japan).

Small interfering RNA experiments

Synovial fibroblasts were transfected with 100 nM non-targeting control small interfering RNA (siRNA; AllStars Negative Control siRNA; Qiagen, Hilden, Germany) or with 50 nM two NLRP3 siRNAs (CIAS1_6 and CIAS1_9; Qiagen), combined with the HiPerFect Transfection Reagent (Qiagen) under serum-free condition, as instructed by the manufacturer. The medium was subsequently replaced, pretreated with SAA for 12 h and stimulated with another 24 h with MSU with medium containing 10% FBS. The cell-culture medium was collected for IL-1β ELISA analysis. In some experiments cells were harvested for total RNA purification after SAA pretreatment and analyzed by semi-quantitative RT-PCR (NLRP3), as described below.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from synovial fibroblasts using the RNeasy total RNA isolation protocol (Qiagen, Crauley, UK) according to the manufacturer's protocol. First-strand cDNA was synthesized from 1 μg of total cellular RNA using an RNA PCR kit (Takara Bio Inc., Otsu, Japan) with random primers. Thereafter, cDNA was amplified using specific primers respectively. The specific primers used were as follows:

NLRP3: forward primer 5'- AAAGAGATGAGCCGAAGTGGG -3' reverse primer 5'-TCAATGCTGTCTTCCTGGCA -3' β-actin; forward primer 5'-GTGGGGCGCCCCAGGCACCA-3' reverse primer 5'-CTCCTTAATGTCACGCACGATTTC-3'.

The product sizes were 79 bp for NLRP3 and 234 bp for β-actin. The thermocycling conditions (35 cycles) 94°C for 60 s and 62°C for 60 s, and 72°C for 60 s.

The amplification of the IL-1-β transcripts was also accomplished on a Light Cycler (Roche Diagnostics, Mannheim, Germany) using specific primers. The housekeeping gene fragment of glyceraldehydes-3-

phosphates dehydrogenase (GAPDH) was used for verification of equal loading.

Cell lysis and immunoblot

Synovial fibroblasts were stimulated with SAA with the indicated concentrations of SAA for 24 h. Cells were washed by ice-cold PBS and lysed with a lysis buffer (1% Nonidet P 40, 50 mM Tris, pH 7.5, 100 mM NaCl, 50 mM NaF, 5 mM EDTA , 20 mM β-glycerophosphate, 1.0 mM sodium orthovanadate, 10 μg/mL aprotinin and 10 μg/mL leupeptin) for 20 minutes at 4°C. Insoluble material was removed by centrifugation at 15,000 × g for 15 minutes at 4°C. The supernatant was saved and the protein concentration was determined using the Bio-Rad protein assay kit (Bio Rad, Hercules, CA, USA). An identical amount of protein (50 μg) for each lysate was subjected to 10% SDS-polyacrylamide gel electrophoresis, and then transferred to a nitrocellulose membrane. Immunoblot analysis using anti-NLRP3, pro-IL-β and β-acitin antibodies was performed with an ECL Western blotting kit (GE Healthcare, BUCKS, UK). In brief, the membrane was probed with primary antibodies and washed and incubated with donkey anti-rabbit secondary antibody conjugated with horseradish peroxidase (1:10,000 diluation; GE Healthcare). After being washed, the membrane was reacted with an ECL advance Western blot detection kit (GE Healthcare). Protein bands were visualized using a lumino-image analyzer (LAS3000; Fujifilm, Toyo, Japan).

Statistical analysis

Differences between groups were examined for statistical significance using Wilcoxon-Mann-Whitney U test. *P*-values less than 0.05 were considered statistically significance.

Results

SAA priming induces mature IL-1β secretion from MSU-treated synoval fibroblasts

SAA has been shown to induce the expression of various proinflammatory cytokines in inflammatory cells. First, we examined whether SAA induces IL-1β secretion from synovial fibroblasts. As shown in Figure 1, SAA is a potent inducer of pro-IL-1β mRNA expression in synovial fibroblasts. However, SAA alone did not induce the secretion of IL-1β, suggesting that SAA alone is not able to provide the signal for the proteolytic cleavage of pro-IL-1β and secretion of mature IL-1β (Figure 2A). In contrast, priming of synovial fibroblasts with SAA resulted in the induction of IL-1β secretion when these cells were subsequently stimulated with MSU (Figure 2A). Immunoblot analysis also indicated that in addition to pro-IL-1β (31 kDa), cleaved mature IL-1β (17 kDa) was also induced by MSU in SAA-primed synovial fibroblasts (Figure 2B).

Endotoxin contamination dose not contribute to the IL-1β induction by SAA/MSA

We next determined whether the induction of IL-1β is a direct effect of SAA or results from contaminating LPS in the SAA preparation. LPS priming induce the IL-1β secretion following MSU stimulation from synovial fibroblasts; however, its IL-1β-inducing ability was lower compared to those of SAA-priming (Figure 3A). Given that most proteins are heat-labile, whereas LPS is heat-resistant, we examined the ability of heat-treated SAA

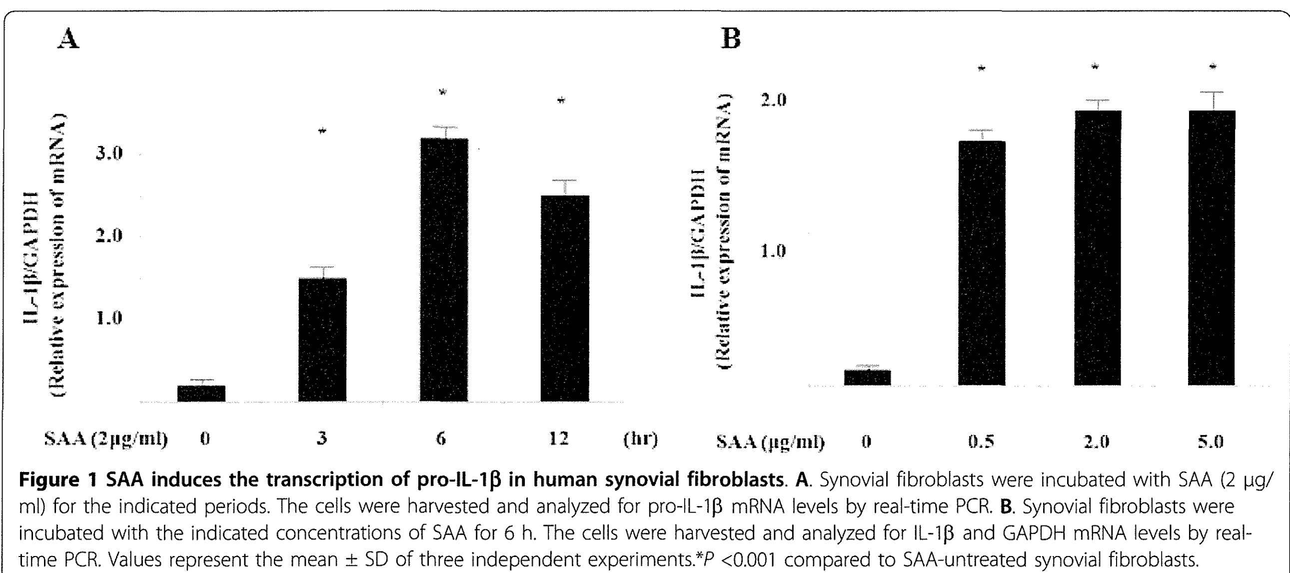

Figure 1 SAA induces the transcription of pro-IL-1β in human synovial fibroblasts. A. Synovial fibroblasts were incubated with SAA (2 μg/ml) for the indicated periods. The cells were harvested and analyzed for pro-IL-1β mRNA levels by real-time PCR. **B**. Synovial fibroblasts were incubated with the indicated concentrations of SAA for 6 h. The cells were harvested and analyzed for IL-1β and GAPDH mRNA levels by real-time PCR. Values represent the mean ± SD of three independent experiments.**P* <0.001 compared to SAA-untreated synovial fibroblasts.

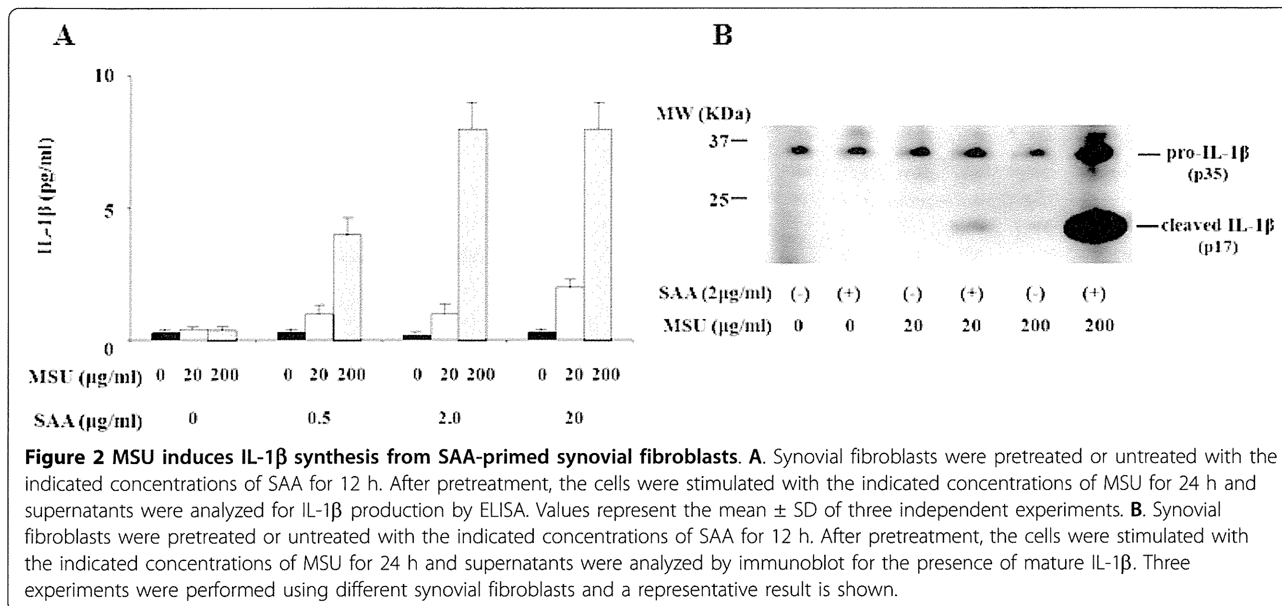

Figure 2 MSU induces IL-1β synthesis from SAA-primed synovial fibroblasts. A. Synovial fibroblasts were pretreated or untreated with the indicated concentrations of SAA for 12 h. After pretreatment, the cells were stimulated with the indicated concentrations of MSU for 24 h and supernatants were analyzed for IL-1β production by ELISA. Values represent the mean ± SD of three independent experiments. **B**. Synovial fibroblasts were pretreated or untreated with the indicated concentrations of SAA for 12 h. After pretreatment, the cells were stimulated with the indicated concentrations of MSU for 24 h and supernatants were analyzed by immunoblot for the presence of mature IL-1β. Three experiments were performed using different synovial fibroblasts and a representative result is shown.

(1 μg/ml) and LPS (500 pg/ml) to stimulate IL-1β secretion from synovial fibroblasts. As shown in Figure 3B, after heating 100°C for 30 minutes, LPS retained its ability to induced MSU-stimulated IL-1β production. In contrast, SAA exposed to 100°C for 30 minutes could not stimulate IL-1β secretion completely as described previously [19]. The endotoxin detected by limulus test was extremely high in LPS-primed synovial fibroblasts-conditioned media, whereas, endotoxin was not detected SAA-primed synovial fibroblast-conditioned media (Figure 3B). These results indicate that the trace amount of LPS in the SAA preparation cannot account for the IL-1β secretion from SAA/MSU-stimulated synovial fibroblasts.

SAA/MSU-induced IL-1β processing is dependent on caspase-1

Previous investigations demonstrated that the inflammatory caspases are cleaved and released along with active IL-1β after activation of the inflammasome [20]. Consistent with these findings, SAA/MSU stimulation activated caspase-1 in synovial fibroblasts (Figure 4A). The caspase-1 dependency for the pro-IL-1β processing was confirmed by addition of the caspase-1 inhibitor z-YVAD-fmk, which completely blocked SAA/MSU-induced IL-1β processing (Figure 4B).

SAA induces NLRP3 expression and cathepsin B release in synovial fibroblasts

The induction of NLRP3 expression is important for inflammasome activation [21]. Therefore, we examined the effects of SAA on NLRP3 expression in synovial fibroblasts. As shown in Figure 5A, a rapid increase in NLRP3 mRNA expression was observed in SAA-stimulated synovial fibroblasts. Also, we analyzed the protein expressions of pro-IL-1β and NLRP3 using the lysates of SAA-stimulated synovial fibroblasts. Immunoblot analysis revealed the protein expressions of pro-IL-1β and NLRP3 were increased by SAA stimulation (Figure 5B). To elucidate the role of NLRP3 in SAA/MSU-induced IL-1β induction, NLRP3 was silenced in synovial fibroblasts using a combination of two small interfering RNAs (siRNA). The siRNA treatments prevented SAA-induced *NLRP3* mRNA expression (Figure 5C). Silencing the *NLRP3* gene reduced SAA/MSU-induced IL-1β secretion, while no inhibition of IL-1β secretion was observed in synovial fibroblasts transfected with negative control siRNA (Figure 5D). Inflammasome activation has been associated with the release of cathepsin B from the cells [22,23]. Therefore, we examined the cell-free culture media for the presence of cathepsin B. As shown in Figure 6, significant secretion of cathepsin B was observed in SAA/MSU-stimulated synovial fibroblasts, as well as SAA-primed synovial fibroblasts.

SAA priming induces IL-1a secretion from MSU-treated synoval fibroblasts

More recently, IL-1α secretion was demonstrated to be required the presence of IL-1β, in which IL-1β directly binds IL-1α as a shuttle [24]. Therefore, we examined whether SAA/MSU stimulation induces IL-1α secretion from rheumatoid synovial fibroblasts. Although neither SAA nor MSU induces IL-1α secretion, SAA priming induces the IL-1α secretion from MSU-stimulated synovial fibroblasts parallel to IL-1β secretion (Figure 7).

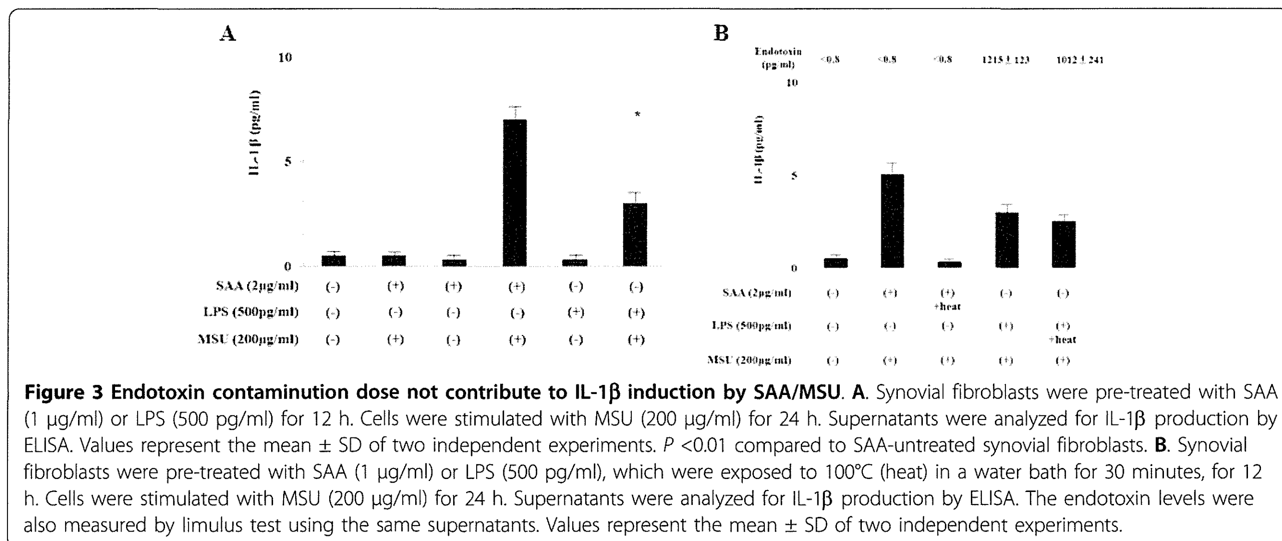

Figure 3 Endotoxin contaminution dose not contribute to IL-1β induction by SAA/MSU. **A**. Synovial fibroblasts were pre-treated with SAA (1 μg/ml) or LPS (500 pg/ml) for 12 h. Cells were stimulated with MSU (200 μg/ml) for 24 h. Supernatants were analyzed for IL-1β production by ELISA. Values represent the mean ± SD of two independent experiments. P <0.01 compared to SAA-untreated synovial fibroblasts. **B**. Synovial fibroblasts were pre-treated with SAA (1 μg/ml) or LPS (500 pg/ml), which were exposed to 100°C (heat) in a water bath for 30 minutes, for 12 h. Cells were stimulated with MSU (200 μg/ml) for 24 h. Supernatants were analyzed for IL-1β production by ELISA. The endotoxin levels were also measured by limulus test using the same supernatants. Values represent the mean ± SD of two independent experiments.

Discussion

Gout is a form of inflammatory arthritis caused by formation of MSU crystals in the synovial tissues of joints [1]. IL-1β has been identified as a pivotal cytokine in gout and MSU crystal-induced inflammation [25]. Recent studies suggested that MSU-mediated NLRP3 inflammasome activation and subsequent IL-1β production in macrophages as key events in initiation of gout [2]. The aim of this study was to determine whether MSU-mediated inflammasome activation could be induced in non-myeloid synovial fibroblasts. A variety of structurally diverse molecules, including ATP, bacterial toxins, crystals, and amyloid proteins, are known to activate the NLRP3 inflammasome leading to IL-1β secretion [26]. Here we found that SAA, which is endogenously induced as an acute phase reactant, sensed the MSU-mediated caspase-1 activation and pro-IL-1β processing. The NLRP3 inflammasome pathway should be pivotal in this SAA/MSU-mediated IL-1β induction, since silencing NLRP3 using siRNA resulted in the abortive IL-1β induction. These data implicate a casual role of SAA in the pathogenesis of MSU-mediated inflammasome activation as well as acute inflammation seen in gouty arthritis.

IL-1β requires cleavage *via* caspase-1 for proper secretion, which is facilitated as a consequence of inflammasome assembly and activation [26]. The NLRP3 inflammasome has emerged as a critical sensor for a number of endogenous mediators, including MSU, that are capable of promoting IL-1β secretion [25]. However, our study demonstrated that MSU alone did not induce caspase-1 activation or IL-1β secretion in human synovial fibroblasts. Because of its pro-IL-1β inducing effect, SAA-priming of synovial fibroblasts could be essential for MSU-induced IL-1β secretion. Our data also suggest that SAA-induced *NLRP3* mRNA expression and cathepsin B secretion may contribute to MSU-mediated NLRP3 activation.

Several lines of evidence indicate that toll-like receptor (TLR) ligands can elicit inflammasome activation [27].

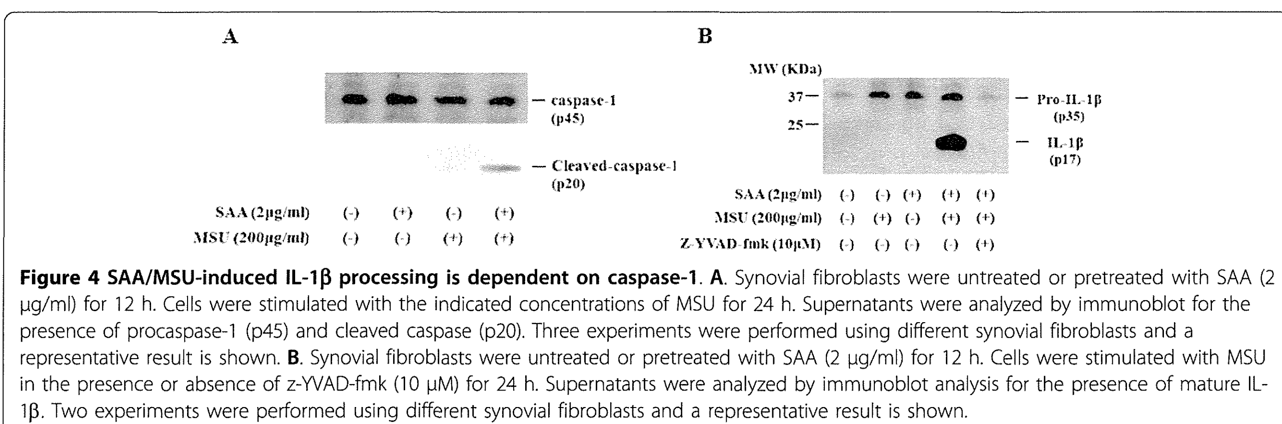

Figure 4 SAA/MSU-induced IL-1β processing is dependent on caspase-1. **A**. Synovial fibroblasts were untreated or pretreated with SAA (2 μg/ml) for 12 h. Cells were stimulated with the indicated concentrations of MSU for 24 h. Supernatants were analyzed by immunoblot for the presence of procaspase-1 (p45) and cleaved caspase (p20). Three experiments were performed using different synovial fibroblasts and a representative result is shown. **B**. Synovial fibroblasts were untreated or pretreated with SAA (2 μg/ml) for 12 h. Cells were stimulated with MSU in the presence or absence of z-YVAD-fmk (10 μM) for 24 h. Supernatants were analyzed by immunoblot analysis for the presence of mature IL-1β. Two experiments were performed using different synovial fibroblasts and a representative result is shown.

Figure 5 SAA induces NLRP3 expression. **A**. Synovial fibroblasts were stimulated with SAA (2 μg/ml) for 12 h. The cells were analyzed for *NLRP3* mRNA by RT-PCR. Three experiments were performed using different synovial fibroblasts and a representative result is shown. **B**. Synovial fibroblasts were stimulated with the indicated concentrations of SAA for 24 h. Cellular lysates were subjected to Western blotting using specific antibodies against NLRP3, pro-IL-1β and β-actin (internal control). Three experiments were performed and a representative result is shown. **C**. Synovial fibroblasts were transfected with non-targeting control siRNA or NLRP3 siRNA for 22 h and subsequently stimulated with SAA (2 μg/ml) for 12 h. The cells were analyzed for *NLRP3* mRNA by RT-PCR. Three experiments were performed using different synovial fibroblasts and a representative result is shown. **D**. The transfected cells as described in C were pretreated with SAA (2 μg/ml) for 12 h and subsequently stimulated with MSU (200 μg/ml) for 24 h. Cell-free culture supernatants were analyzed for IL-1β by ELISA. Values represent the mean ± SD of three independent experiments. *P <0.01 compared to control siRNA transfected synovial fibroblasts.

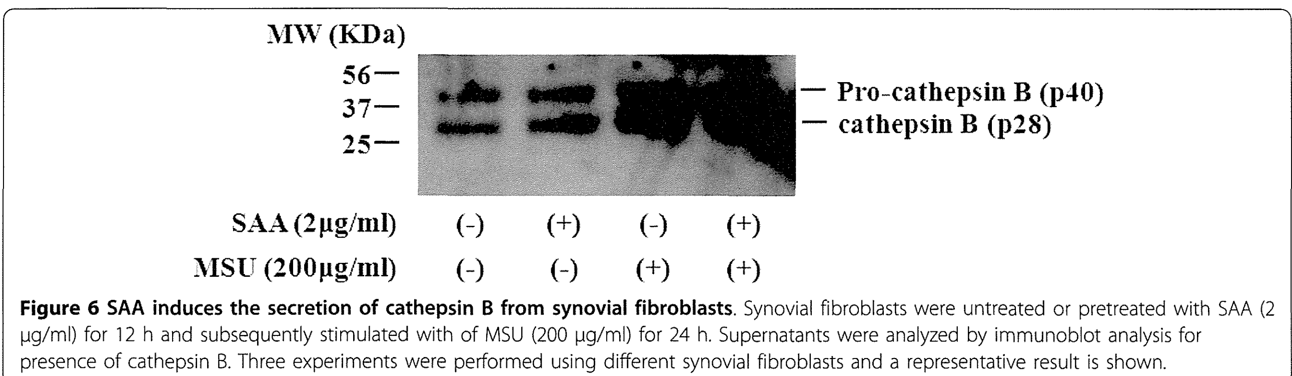

Figure 6 SAA induces the secretion of cathepsin B from synovial fibroblasts. Synovial fibroblasts were untreated or pretreated with SAA (2 μg/ml) for 12 h and subsequently stimulated with of MSU (200 μg/ml) for 24 h. Supernatants were analyzed by immunoblot analysis for presence of cathepsin B. Three experiments were performed using different synovial fibroblasts and a representative result is shown.

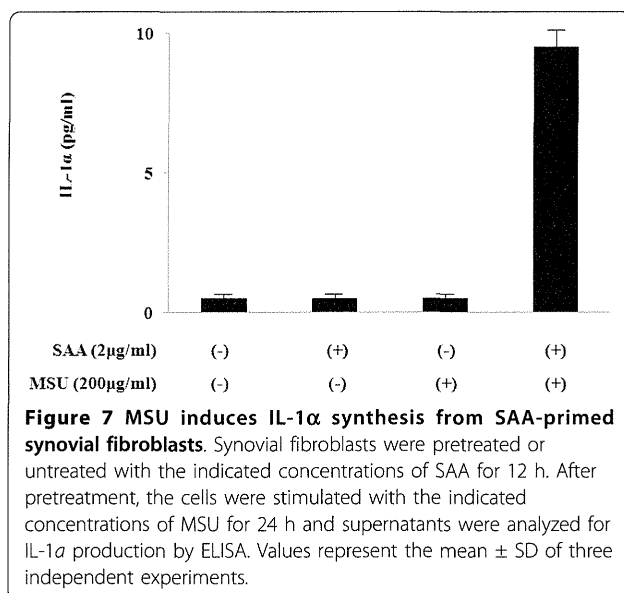

Figure 7 MSU induces IL-1α synthesis from SAA-primed synovial fibroblasts. Synovial fibroblasts were pretreated or untreated with the indicated concentrations of SAA for 12 h. After pretreatment, the cells were stimulated with the indicated concentrations of MSU for 24 h and supernatants were analyzed for IL-1*a* production by ELISA. Values represent the mean ± SD of three independent experiments.

Our findings suggest that SAA, a non-bacterial endogenous product, is sufficient to trigger caspase-1 activation and IL-1β processing in response to MSU, providing a mechanism for activation of the NLRP3 inflammasome in human synovial tissues. Endogenous molecules may be the first signal to prime the activation of the NLRP3 inflammasome, resulting in cooperative signaling [28]. The second signal is provided by stimuli that specifically activate NLRP3 and leads to caspase 1 activation and IL-1β processing [28]. Our results suggest that an endogenous proinflammatory molecule, SAA, could be the first signal to prime the activation of the NLRP3 inflammasome.

Conclusion

Our data indicate that SAA induced MSU-mediated NLRP3 inflammasome activation and post-translational processing of IL-1β in human synovial fibroblasts. These findings highlight the potential role of SAA, a highly sensitive acute phase reactant, in the triggering of MSU-mediated acute synovial inflammation. The innate immune systems, including TLRs, are thought to be essentially involved in inflammasome-mediated inflammation [27]. However, our data show that interaction of an endogenous and non-bacterial acute phase protein, SAA, and MSU crystals synergistically enhance the inflammatory response by activating the inflammasome pathway. These findings provide a new insight into the mechanisms underlying acute gout.

Abbreviations

ATP: adenosine triphosphate; ASC: apoptosis-associated speck-like protein containing CARD; IL-1β: interleukin-1β; NLRP3: Nod-like receptor family: pyrin domain containing 3; MSU: monosodium urate; SAA: serum amyloid A; TLR: toll-like receptor.

Acknowledgements

This work was supported by a Grant-in-Aid for Research on intractable diseases from Ministry of Health, Labour and Welfare of Japan, "Study group of national-wide survey for FMF in Japan"

Author details

[1]Department of Rheumatology, Nagasaki Medical Center, Kubara 2-1001-1, Omura, Nagasaki 856-8562, Japan. [2]Orthopedic Surgery, Nagasaki Medical Center, Kubara 2-1001-1, Omura, Nagasaki 856-8562, Japan. [3]Clinical Research Center, Nagasaki Medical Center, Kubara 2-1001-1, Omura, Nagasaki 856-8562, Japan. [4]Department of Rheumatology, Nagasaki University Hospital, Sakamoto 1-7-1, Nagasaki 852-8201, Japan. [5]Department of Rheumatology, NTT West Japan Hospital, Shinyashiki 1-17 Kumamoto 862-8655, Japan.

Authors' contributions

KM, AK, TT, KS, MI, YM and YJ carried out cell culture and biochemical analysis. TK, YI, TM and MN participated in the design of the study and performed the statistical analysis. AK, SM, HI and TN conceived the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 28 December 2011 Revised: 4 April 2012
Accepted: 18 May 2012 Published: 18 May 2012

References

1. Lioté F, Ea HK: **Gout: update on some pathogenic and clinical aspects.** *Rheum Dis Clin North Am* 2006, **32**:295-311.
2. Martinon F: **Mechanisms of uric acid crystal-mediated autoinflammation.** *Immunol Rev* 2010, **233**:218-232.
3. Burns K, Martinon F, Tschopp J: **New insights into the mechanism of IL-1beta maturation.** *Curr Opin Immunol* 2003, **15**:26-30.
4. Martinon F, Burns K, Tschopp J: **The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta.** *Mol Cell* 2002, **10**:417-426.
5. Martinon F, Pétrilli V, Mayor A, Tardivel A, Tschopp J: **Gout-associated uric acid crystals activate the NALP3 inflammasome.** *Nature* 2006, **440**:237-241.
6. Shi Y, Evans JE, Rock KL: **Molecular identification of a danger signal that alerts the immune system to dying cells.** *Nature* 2003, **425**:516-521.
7. Matzinger P: **The danger model: a renewed sense of self.** *Science* 2002, **296**:301-305.
8. Ruschen S, Lemm G, Warnatz H: **Spontaneous and LPS-stimulated production of intracellular IL-1 beta by synovial macrophages in rheumatoid arthritis is inhibited by IFN-gamma.** *Clin Exp Immunol* 1989, **76**:246-251.
9. Dinarello CA: **Immunological and inflammatory functions of the interleukin-1 family.** *Annu Rev Immunol* 2009, **27**:519-550.
10. Martinon F, Tschopp J: **NLRs join TLRs as innate sensors of pathogens.** *Trends Immunol* 2005, **26**:447-454.
11. Liu-Bryan R, Pritzker K, Firestein GS, Terkeltaub R: **TLR2 signaling in chondrocytes drives calcium pyrophosphate dihydrate and monosodium urate crystal-induced nitric oxide generation.** *J Immunol* 2005, **174**:5016-5023.
12. Liu-Bryan R, Scott P, Sydlaske A, Rose DM, Terkeltaub R: **Innate immunity conferred by Toll-like receptors 2 and 4 and myeloid differentiation factor 88 expression is pivotal to monosodium urate monohydrate crystal-induced inflammation.** *Arthritis Rheum* 2005, **52**:2936-2946.
13. Pope RM, Tschopp J: **The role of interleukin-1 and the inflammasome in gout: implications for therapy.** *Arthritis Rheum* 2007, **56**:3183-3188.
14. Chen CJ, Shi Y, Hearn A, Fitzgerald K, Golenbock D, Reed G, Akira S, Rock KL: **Rock. MyD88-dependent IL-1 receptor signaling is essential for gouty inflammation stimulated by monosodium urate crystals.** *J Clin Invest* 2006, **116**:2262-2271.
15. Su SB, Gong W, Gao JL, Shen W, Murphy PM, Oppenheim JJ, Wang JM: **A seven-transmembrane, G protein-coupled receptor, FPRL1, mediates the**

chemotactic activity of serum amyloid A for human phagocytic cells. *J Exp Med* 1999, **189**:395-402.
16. Vallon R, Freuler F, Desta-Tsedu N, Robeva A, Dawson J, Wenner P, Engelhardt P, Boes L, Schnyder J, Tschopp C, Urfer R, Baumann G: **Serum amyloid A (apoSAA) expression is up-regulated in rheumatoid arthritis and induces transcription of matrix metalloproteinases.** *J Immunol* 2001, **166**:2801-2807.
17. Halle A, Hornung V, Petzold GC, Stewart CR, Monks BG, Reinheckel T, Fitzgerald KA, Latz E, Moore KJ, Golenbock DT: **The NALP3 inflammasome is involved in the innate immune response to amyloid-beta.** *Nat Immunol* 2008, **9**:857-865.
18. Niemi K, Teirilä L, Lappalainen J, Rajamäki K, Baumann MH, Öörni K, Wolff H, Kovanen PT, Matikainen S, Eklund KK: **Serum amyloid A activates the NLRP3 inflammasome via P2X7 receptor and a cathepsin B-sensitive pathway.** *J Immunol* 2011, **186**:6119-6128.
19. Cai H, Song C, Endoh I, Goyette J, Jessup W, Freedman SB, McNeil HP, Geczy CL: **Serum amyloid A induces monocyte tissue factor.** *J Immunol* 2007, **178**:1852-1860.
20. Franchi L, Eigenbrod T, Muñoz-Planillo R, Nuñez G: **The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis.** *Nat Immunol* 2009, **10**:241-247.
21. Lamkanfi M, Dixit VM: **Inflammasomes: guardians of cytosolic sanctity.** *Immunol Rev* 2009, **227**:95-105.
22. Qu Y, Franchi L, Nunez G, Dubyak GR: **Nonclassical IL-1 beta secretion stimulated by P2X7 receptors is dependent on inflammasome activation and correlated with exosome release in murine macrophages.** *J Immunol* 2007, **179**:1913-925.
23. Hornung V, Bauernfeind F, Halle A, Samstad EO, Kono H, Rock KL, Fitzgerald KA, Latz E: **Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization.** *Nat Immunol* 2008, **9**:847-856.
24. Fettelschoss A, Kistowska M, LeibundGut-Landmann S, Beer HD, Johansen P, Senti G, Contassot E, Bachmann MF, French LE, Oxenius A, Kündig TM: **Inflammasome activation and IL-1β target IL-1α for secretion as opposed to surface expression.** *Proc Natl Acad Sci USA* 2011, **108**:18055-18060.
25. Shi Y, Mucsi AD, Ng G: **Monosodium urate crystals in inflammation and immunity.** *Immunol Rev* 2010, **233**:203-217.
26. Latz E: **The inflammasomes: mechanisms of activation and function.** *Curr Opin Immunol* 2010, **22**:28-33.
27. Meylan E, Tschopp J, Karin M: **Intracellular pattern recognition receptors in the host response.** *Nature* 2006, **442**:39-44.
28. Ogura Y, Sutterwala FS, Flavell RA: **The inflammasome: first line of the immune response to cell stress.** *Cell* 2006, **126**:659-662.

doi:10.1186/ar3849
Cite this article as: Migita *et al.*: Serum amyloid A triggers the mosodium urate -mediated mature interleukin-1β production from human synovial fibroblasts. *Arthritis Research & Therapy* 2012 **14**:R119.

Submit your next manuscript to BioMed Central and take full advantage of:

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at
www.biomedcentral.com/submit

Familial Mediterranean Fever in Japan

*Kiyoshi Migita, MD, Ritei Uehara, MD, Yoshikazu Nakamura, MD, Michio Yasunami, MD, Ayako Tsuchiya-Suzuki, MD, Masahide Yazaki, MD, Akinori Nakamura, MD, Junya Masumoto, MD, Akihiro Yachie, MD, Hiroshi Furukawa, MD, Hiromi Ishibashi, MD, Hiroaki Ida, MD, Kazuko Yamazaki, MD, Atsushi Kawakami, MD, and Kazunaga Agematsu, MD**

Abstract: Familial Mediterranean fever (FMF) is a hereditary autoinflammatory disease that is prevalent in Mediterranean populations. While it is considered a rare disease in the rest of world, a significant number of FMF patients have been reported in East Asia, including Japan. Our aim was to determine the prevalence of FMF in Japan and elucidate the clinical and genetic features of Japanese patients. A primary nationwide survey of FMF was conducted between January and December 2009. Hospitals specializing in pediatrics and hospitals with pediatric, internal medicine, and rheumatology/allergy departments were asked to report all patients with FMF during the survey year. The estimated total number of Japanese FMF patients was 292 (95% confidence interval, 187–398 people). We evaluated the clinical and genetic profiles of Japanese patients from the data obtained in a secondary survey of 134 FMF patients. High-grade fever was observed in 95.5%, chest pain (pleuritis symptoms) in 36.9%, abdominal pain (peritonitis symptoms) in 62.7%, and arthritis in 31.3%. Of the patients profiled, 25.4% of patients experienced their first attack before 10 years of age, 37.3% in their teens, and 37.3% after age 20 years. Colchicine was effective in 91.8% of patients at a relatively low dose (mean dose, 0.89 ± 0.45 mg/d). AA amyloidosis was confirmed in 5 patients (3.7%). Of the 126 patients studied, 109 (86.5%) were positive for 1 or more genetic mutations and 17 (13.5%) had no mutation detected. Common Mediterranean fever gene (MEFV) mutations were E148Q/M694I (19.8%) and M694I/normal (12.7%). The differences in the prevalence of peritonitis, pleuritis, and a family history of FMF were statistically significant between FMF patients with MEFV exon 10 mutations compared with those without exon 10 mutations.

In conclusion, a significant number of patients with FMF exist in Japan. Although Japanese patients with FMF are clinically or genetically different from Mediterranean patients, the delay in diagnosis is an issue that should be resolved.

From the Clinical Research Center (KM, H Ishibashi), Nagasaki Medical Center, Omura, Nagasaki; Institute of Tropical Medicine (NEKKEN) (M Yasunami), Nagasaki University, Nagasaki; Department of Rheumatology (AK), Nagasaki University Hospital, Nagasaki; Department of Public Health (RU, YN), Jichi Medical University, Tochigi; Department of Medicine (Neurology and Rheumatology) (ATS, M Yazaki, AN), Department of Pathology (JM), Department of Infection and Host Defense (KY, KA), Shinshu University School of Medicine, Matsumoto; Department of Pediatrics (AY), School of Medicine, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kanazawa; Clinical Research Center (HF), Sagamihara National Hospital, National Hospital Organization, Sagamihara, Kanagawa; Department of Rheumatology (H Ida), Kurume University School of Medicine, Kurume; Japan.

*Study Group of Familial Mediterranean Fever for Research on Intractable Diseases, Health and Labour Science Research Grants, Ministry of Health, Labour and Welfare of Japan.

This work was supported by a grant-in-aid for research on intractable diseases from Ministry of Health, Labour and Welfare of Japan. The authors have no conflicts of interest to disclose.

Reprints: Kiyoshi Migita, MD, Clinical Research Center, NHO Nagasaki Medical Center, Kubara 2-1001-1, Omura 856-8652, Japan (e-mail: migita@nmc.hosp.go.jp).

Copyright © 2012 by Lippincott Williams & Wilkins

ISSN: 0025-7974
DOI: 10.1097/MD.0b013e318277cf75

(*Medicine* 2012;91: 337–343)

Abbreviations: AA = amyloid A, CI = confidence interval, FMF = familial Mediterranean fever, MEFV = Mediterranean fever, PCR = polymerase chain reaction, SAA = serum amyloid A.

INTRODUCTION

Familial Mediterranean Fever (FMF) is an inherited autoinflammatory disease that is observed in Mediterranean populations, such as Armenians, Arabs, non-Ashkenazi Jews, and Turks.[11,47] The disease is characterized by recurrent febrile episodes and inflammation in the form of sterile polyserositis.[5] The gene responsible for FMF, MEFV, encodes a protein called pyrin/marenostrin and is expressed mainly in neutrophils and monocytes.[13,21] To date, 200 mutations or polymorphisms in the MEFV gene have been associated with the FMF phenotype.[28] The prevalence of FMF varies from 1:400-1000 in Turkey,[9,47] 1:1000 (depending on the ethnic group) in Israel,[10] and 1:500 in Armenia.[35] The various manifestations of FMF in different populations could be caused by a diverse repertoire of mutations specific to their ethnic background.[7] For example, patients carrying exon 2 mutations (such as E148Q) present a milder phenotype.[7] In contrast, patients carrying M694V or M694I mutations are prone to more severe disease.[44] Where the disease is common the diagnosis of FMF is principally based on clinical tests, whereas in countries where FMF is rare, a genetic test is useful.[2] The diagnostic power of the colchicine response, where an FMF patient is expected to respond to colchicine, is still important when monitoring atypical FMF cases.[6]

The genetic homogeneity of Japan has been preserved by national geographic borders, and there has been little inward migration since ancient times. FMF was previously thought to affect people mainly from Mediterranean populations, and was considered a rare disease in Japan. However, a significant number of FMF patients with MEFV gene mutations have been reported in Japan since the identification of the MEFV gene.[22,30,36,38,41,42,46] One severe complication of FMF is the development of amyloid A (AA) amyloidosis.[17] The effect of MEFV genotypes, especially when the M694V mutation is homozygous, is evident in FMF patients with AA amyloidosis.[4] In Japanese patients with rheumatoid arthritis, the SAA1.3 allele was shown to be a risk factor of AA amyloidosis.[31] However, there is a strong positive association of the SAA1.1 allele and M694V homozygosity of the MEFV gene as risk factors for AA amyloidosis in white FMF patients.[16]

Since 1972, the Ministry of Health and Welfare of Japan has promoted research to determine the causes of intractable diseases of unknown etiology.[32] To investigate epidemiologic features of disease (prevalence, age distribution, and clinical phenotypes), the Research Committee on the Epidemiology of Intractable Diseases conducted several surveys in cooperation with various disease research committees.[18] In 2009 the Ministry of Health,