Mod Rheumatol (2013) 23:210–217
DOI 10.1007/s10165-012-0737-3

REVIEW ARTICLE

Tumor necrosis factor receptor-associated periodic syndrome (TRAPS) in Japan: a review of the literature

Masakazu Washio · Teruaki Nakano · Yasushi Kawaguchi · Kae Takagi · Chikako Kiyohara · Hiroshi Tsukamoto · Shoji Tokunaga · Takahiko Horiuchi

Received: 19 March 2012 / Accepted: 31 July 2012 / Published online: 24 August 2012

Abstract Tumor necrosis factor receptor-associated periodic syndrome (TRAPS) is a dominantly inherited autoinflammatory syndrome that is characterized by recurrent episodes of fever attacks associated with rashes, abdominal pain, myalgia, conjunctivitis, chest pain, and arthralgia. Some patients have severe abdominal pain leading to abdominal surgery. Most reported cases of TRAPS involve patients of European ancestry, but there have been nine reports of patients with TRAPS in Japan. Here, we review these nine case reports. Reported *TNFRSF1A* gene mutations in these nine index patients were C70S, T61I, C70G, C30Y, C30R, N101K, and N25D. Fever (100 %) was seen in all 23 cases. Most patients developed rash (erythema) (84.6 %) and arthralgia (73.3 %), and half suffered from myalgia (54.5 %) and abdominal pain (50.0 %). Although one-half of the patients suffered from abdominal pain, none underwent surgery. In contrast, only a small percentage of patients suffered from chest pain (20.0 %), conjunctivitis (20.0 %), and headache (10.0 %). Almost all cases (95.7 %) concerned patients whose relatives suffered from periodic fever. These findings suggest that the clinical features of Japanese TRAPS patients may be milder than those of patients in Western countries.

Keywords TRAPS · Autoinflammatory disease · *TNFRSF1A* gene mutation · Japan

M. Washio (✉)
St. Mary's College, 422 Tsubukuhonmachi,
Kurume, Fukuoka 830-8558, Japan
e-mail: washio@st-mary.ac.jp

T. Nakano
St. Mary's Hospital, Kurume, Fukuoka, Japan

Y. Kawaguchi · K. Takagi
Tokyo Women's Medical University, Shinjuku-ku, Tokyo, Japan

C. Kiyohara · H. Tsukamoto · T. Horiuchi
Kyushu University Graduate School of Medical Sciences,
Higashi-ku, Fukuoka, Japan

S. Tokunaga
Kyushu University Hospital, Higashi-ku, Fukuoka, Japan

Introduction

Tumor necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS), formerly known as Familial Hibernian Fever [1], is an inherited autoinflammatory syndrome that is caused by mutations in the TNF receptor type 1 (*TNFRSF1A*), the gene encoding for the 55-kDa receptor for TNF [2–6]. The *TNFRSF1A* gene mutations were first thought to be associated with a deregulation of the shedding of TNFRSF1A [2], and in 2006 Lobito and colleagues [7] reported that the mutations may spontaneously induce alternative signaling, independent of binding TNF-α. Recently, the concerted pro-inflammatory action of cell-surface wild-type and accumulated intracellular mutant TNF receptors has been reported [8]. Since TRAPS was first proposed as a genetic diagnosis, only those patients with demonstrable TNF receptor mutations should be included as having TRAPS [2–6].

More than 100 different mutations have been reported for patients with TRAPS in INFEVERS, a mutational database accessible on the World Wide Web at http://fmf.igh.cnrs.fr/infevers. However, a few *TNFRSF1A* gene mutations are also found in about 1 % of the general population [9]. The *TNFRSF1A* gene mutations that are present in unaffected individuals are considered to be low-penetrance gene mutations [10].

TRAPS is characterized by recurrent episodes of fever attacks associated with rash, abdominal pain, myalgia, conjunctivitis, chest pain, and arthralgia [2–6]. Hull and colleagues [3] reported the clinical characteristics of 50 genetically confirmed American TRAPS patients (i.e., 47 of European ancestry, 2 Puerto Ricans, and 1 African American). Common symptoms associated with periodic fever were myalgia (98.0 %), conjunctivitis/periorbital edema (90.0 %), abdominal pain (88.0 %), rash (86.0 %), arthralgia (84.0 %), and pleuritis (54.0 %). Among these 50 patients, 26 (52.0 %) had abdominal pain which led to abdominal surgery. Pelagatti and colleagues [11] reported the clinical profiles of 11 Italian patients with TRAPS, noting that the recurrent fever attacks were associated with abdominal pain (81.8 %), arthralgia (63.6 %), myalgia (63.6 %), rash (54.4 %), headache (54.4 %), chest pain/pleuritis (27.2 %), and conjunctivitis (9.0 %). Although these authors reported that arthralgia (63.6 %) was frequently associated with periodic fever, arthritis (27.2 %) was less common [11].

TRAPS was originally described as occurring among Irish and Scottish descendants [1], and most reported patients with TRAPS are of Northern European ancestry [2–6], although any ethnic group may be afflicted in this disease [2–6].

We have reported Japanese TRAPS families with the C70G mutation [12] and C30R mutation [13] of the *TNFRSF1A* gene. However, including our reports [12, 13], there have been only nine reported cases of patients with genetically confirmed TRAPS in Japan [12–20]. Moreover, to our knowledge, no TRAPS patients have been reported in East Asian countries, with the exception of Japan. In order to clarify the characteristics of TRAPS in the East Asian population, as well as to explore the modified diagnostic criteria for Japanese TRAPS patients, we reviewed the published case reports on Japanese patients with genetically confirmed TRAPS.

Japanese patients with TRAPS

Table 1 presents a summary of the reported cases of Japanese patients with TRAPS and information on their families [12–20]. Seven different mutations of the *TNFRSF1A* gene were reported in these nine Japanese patients with TRAPS: C70S (T295A) [14], T61I (C269T) [15, 20], C70G (T295G) [12], C30Y (G176A) [16, 19], C30R (T175C) [13], N101K (C390G) [17], and N25D (A160G) [18].

All mutations reported among these Japanese patients are located in the exon regions. Among the nine reports of gene mutations of *TNFRSF1A*, six reports demonstrated that family members also had gene mutations as well as clinical symptoms [12–16, 20]. One report showed that family members had episodes of periodic fever [18, 19], but no genetic analysis was performed [18, 19]. Another paper reported that a patient with TRAPS had no family history of the disease [17].

Three of the nine index cases (30.0 %) and six of 23 patients (26.1 %) had been misdiagnosed as having juvenile idiopathic arthritis (JIA) or adult onset Still's disease prior to the diagnosis of TRAPS being established [13, 16, 19] (see remarks in Table 1). All of these patients had a family history of periodic fever [13, 16, 19].

As shown in Table 2, fever was observed in all Japanese index cases [12–20]. Almost 90 % of all index patients developed rash (erythema) [12–18, 20], and nearly 80 % suffered from arthralgia [12, 14–19]; only half suffered from myalgia (55.6 %) [15, 16, 18–20]. In contrast, only a small percentage of Japanese index cases suffered from abdominal pain (33.3 %) [16, 19, 20], chest pain (22.2 %) [16, 19], conjunctivitis (22.2 %) [14, 15], and headache (11.1 %) [18].

In almost 70 % of the Japanese index cases, fever and rash improved after glucocorticoid therapy (66.7 %) [13–17, 19]. With the exception of two index cases who had no chance to use glucocorticoid [12, 18], fever and rash improved after glucocorticoid therapy in 6 out of 9 Japanese index cases with TRAPS (77.8 %) [13–17, 19]. All Japanese index cases but one (88.9 %) had patients with periodic fever occurring among their relatives [12–16, 18–20].

Table 2 also presents the summary of the clinical symptoms of Japanese index cases and TRAPS patients in their families [12–20]. Fever was seen in all cases [12–20], and most patients presented with rash (erythema) (84.6 %) [12–18, 20] and arthralgia (73.3 %) [12–19], and half suffered from myalgia (54.5 %) [15, 16, 18–20] and abdominal pain (50.0 %) [15, 16, 19, 20]. Although half of the Japanese TRAPS patients suffered from abdominal pain, none underwent surgery [15, 16, 19, 20]. In contrast, only a small percentage of patients suffered from chest pain (20.0 %) [16, 19], conjunctivitis (20.0 %) [14, 15], and headache (10.0 %) [17]. Among the ten patients who received glucocorticoid therapy, 80 % were responsive [13–17, 19]. All cases but one (95.7 %) had relatives who suffered from periodic fever attacks [12–16, 18–20].

Discussion

Hereditary periodic fevers are a group of inherited systemic disorders characterized by episodes of fever with localized inflammation that often affects serosal membranes, joints, and skin [4]. The clinical features of periodic fevers are episodes of fever and localized inflammation, which can include abdominal pain, pleuritic chest pain, arthritis or

Table 1 Reported cases of Japanese patients with TRAPS and their families

References	Age (years) and sex	Kinship	Age at onset (years)	*TNFRSF1A* gene mutation	Fever	Abdominal pain	Myalgia	Rash (erythema)	Conjunctivitis (periorbital edema)	Chest pain (pleuritis)	Arthralgia	Other symptoms	Response to glucocorticoids	Remarks
Kusuhara et al. [14]	14F	Proband	0 (2 months)	C70S (T295A)	Positive	Negative	Negative	Positive	Positive	Negative	Positive	Negative	Responder	Her 17-year-old sister was a mutation carrier with C70S (T295A)
	48F	Mother	Childhood	C70S (T295A)	Positive	NA	NA	NA	NA	NA	NA	NA	NA	Similar symptoms were observed as the proband
	45M	Maternal uncle	NA	Not examined	NA	NA	NA	NA	NA	NA	NA	NA	NA	Similar symptoms were observed as the proband
	Deceased (85F)	Maternal great grandmother	NA	Not examined	NA	NA	NA	NA	NA	NA	NA	NA	NA	Similar symptoms were observed as the proband
	Deceased (57M)	Maternal grandfather	NA	Not examined	NA	NA	NA	NA	NA	NA	NA	NA	NA	Similar symptoms were observed as the proband
Ida et al. [15]	27F	Proband	6 or 7	T61I (C269T)	Positive	Negative	Positive	Positive	Positive	Negative	Positive	Negative	Responder	She was diagnosed with SLE at the age of 21 years. Mother, 3 sisters, 2 brothers, and 1 nephew were mutation carriers with T61I (C269T)
	18F	Niece	NA	T61I (C269T)	Positive	Positive	NA	Positive	NA	NA	Negative	NA	NA	Established the diagnosis of TRAPS but did not fulfil the criteria for SLE
	6M	Nephew	NA	T61I (C269T)	Positive	Positive	NA	Negative	NA	NA	Negative	NA	NA	Established the diagnosis of TRAPS but did not fulfil the criteria for SLE
	4F	Niece	NA	T61I (C269T)	Positive	Positive	NA	Positive	NA	NA	Positive	NA	NA	Established the diagnosis of TRAPS but did not fulfil the criteria for SLE
Horiuchi et al. [12]	32M	Proband	Childhood	C70G (T295G)	Positive	Negative	Negative	Positive	Negative	Negative	Positive	Negative	No chance to use	
	87M	Grandfather	NA	C70G (T295G)	NA	NA	NA	NA	NA	NA	NA	NA	NA	Similar symptoms were observed as the proband
	55M	Father	NA	C70G (T295G)	NA	NA	NA	NA	NA	NA	NA	NA	NA	Similar symptoms were observed as the proband
	33M	Brother	NA	Not examined	NA	NA	NA	NA	NA	NA	NA	NA	NA	Similar symptoms were observed as the proband

Table 1 continued

References	Age (years) and sex	Kinship	Age at onset (years)	*TNFRSF1A* gene mutation	Fever	Abdominal pain	Myalgia	Rash (erythema)	Conjunctivitis (periorbital edema)	Chest pain (pleuritis)	Arthralgia	Other symptoms	Response to glucocorticoids	Remarks
Manki et al. [16]	10M	Proband	0 (6 months)	C30Y (G176A)	Positive	Positive	Positive	Positive	Negative	Positive	Positive	Negative	Responder	He was misdiagnosed as systemic JIA based on the ILAR criteria (prolonged spike-fever, skin rash, arthritis, and pericarditis) at 3 years of age
	7F	Sister	3	C30Y (G176A)	Positive	Positive	NA	NA	NA	NA	Positive	NA	Responder	She was suspected of having systemic JIA at 3 years of age although the clinical symptoms did not fulfill the ILAR criteria
	38M	Mother	28	Not examined	Positive	NA	Positive	NA	NA	NA	Positive	NA	NA	She had recurrent fever, arthralgia, and myalgia after the delivery of a daughter
Takagi et al. [13]	36F	Proband	22	C30R (T175C)	Positive	Negative	Negative	Positive	Negative	Negative	Negative	Negative	Responder	She had experienced periodic high-grade fever after the birth of her elder son. Fever was often accompanied with skin rash and lymphadenopathy. She was diagnosed as having adult onset Still's disease before the diagnosis of TRAPS
	11M	Son	0 (7 months)	C30R (T175C)	Positive	Negative	Negative	Positive	Negative	Negative	Positive	Negative	Responder	Tentatively diagnosed as having JIA before the diagnosis of TRAPS
	9M	Son	3	C30R (T175C)	Positive	NA	NA	NA	NA	NA	NA	NA	Non-responder	Tentatively diagnosed as having JIA before the diagnosis of TRAPS. Fever did not respond to either corticosteroid or non-steroidal anti-inflammatory drugs, but disappeared for several months independent of treatment
Nakamura et al. [17]	17F	Proband	17	N101K (C390G)	Positive	Negative	Negative	Positive	Negative	Negative	Positive	Negative	Responder	Sporadic case. No family history of TRAPS was described

Table 1 continued

References	Age (years) and sex	Kinship	Age at onset (years)	*TNFRSF1A* gene mutation	Fever	Abdominal pain	Myalgia	Rash (erythema)	Conjunctivitis (periorbital edema)	Chest pain (pleuritis)	Arthralgia	Other symptoms	Response to glucocorticoids	Remarks
Nakamura and Tokura [18]	29F	Proband	29	N25D (A160G[a])	Positive	Negative	Positive	Positive	Negative	Negative	Positive	Positive[b]	No chance to use	Her son had fever one to three times a month since he was 2 months old. Her grandfather also had episodes of periodic fever for a long time
Kai et al. [19]	21F	Proband	Childhood	C30Y (G176A)	Positive	Positive	Positive	Negative	Negative	Positive	Positive	Negative	Responder	She was misdiagnosed as systemic JIA at 11 years of age. Her newborn baby had unexplained fever and the plan was to examine gene mutations related to TRAPS. Her father had died with amyloidosis
Ohmori et al. [20]	16F	Proband	15	T61I (C269T)	Positive	Positive	Positive	Positive	Negative	Negative	Negative	Positive[c]	Non-responder	Oral prednisolone (20 mg/day) could not reduce inflammation but anti-TNF antibody and infliximab did so. Her mother and grandfather were diagnosed as TRAPS patients with similar episodes of inflammation and T61I mutation

TRAPS tumor necrosis factor receptor-associated periodic syndrome, *F* female, *M* male, *NA* not available, *TNFRSF1A* TNF receptor superfamily 1A, *SLE* systemic lupus erythematosus, *JIA* juvenile idiopathic arthritis, *ILAR* International League Against Rheumatism

[a] Changed from A73G [14] to A160G according to a mutational database accessible on the World Wide Web at http://fmf.igh.cnrs.fr/infevers

[b] Headache

[c] Dyspnea, localized edema

Table 2 Summary of clinical symptoms of Japanese TRAPS patients

Patients with TRAPS	Female sex (%)	Age at onset (years)	*TNFRSF1A* gene mutation (%)	Fever	Abdominal pain	Myalgia	Rash (erythema)	Conjunctivitis (periorbital edema)	Chest pain (pleuritis)	Arthralgia	Headache	Response to glucocorticoids	Family history of periodic fever
Index cases [12–20]	$n = 9$	$n = 9$	$n = 9$	$n = 9$	$n = 9$	$n = 9$	$n = 9$	$n = 9$	$n = 9$	$n = 9$	$n = 9$	$n = 9$	$n = 9$
	7 (77.8 %)	0 (2 months) to 29	9 (100 %)	9 (100 %)	3 (33.3 %)	5 (55.6 %)	8 (88.9 %)	2 (22.2 %)	2 (22.2 %)	7 (77.8 %)	1 (11.1 %)	6 (66.7 %)[b]	8 (88.9 %)
Index cases and patients in their family [12–20]	$n = 23$	$n = 14$	$n = 23$	$n = 17$	$n = 14$	$n = 11$	$n = 13$	$n = 10$	$n = 10$	$n = 15$	$n = 10$	$n = 12$	$n = 23$
	12 (52.2 %)	0 (2 months) to 29	18 (78.3 %)[a]	17 (100 %)	7 (50.0 %)	6 (54.5 %)	11 (84.6 %)	2 (20.0 %)	2 (20.0 %)	11 (73.3 %)	1 (10.0 %)	8 (66.7 %)[c]	22 (95.7 %)

[a] 7 of 9 patients (77.8 %) who received glucocorticoids were responders

[b] 5 family members were diagnosed as having TRAPS with clinical symptoms and family histories

[c] 8 of 10 patients (80.0 %) who received glucocorticoids were responders

arthralgia, myalgia, rashes (erythematous macular rash), and conjunctivitis [3, 4]. Periodic fevers can be characterized according to their mode of inheritance—recessively or dominantly inherited [3, 4], with familial Mediterranean fever (FMF) and hyperimmunoglobulinemia D with periodic fever syndrome (HIDS) composing the group of recessively inherited periodic fevers and TRAPS, familial cold autoinflammatory syndrome (FCAS), Muckle–Wells syndrome (MWS), and neonatal-onset multisystem inflammatory disease [NOMID; which is also known as chronic infantile neurological cutaneous and articular syndrome (CINCA)] composing the group of dominantly inherited periodic fevers [3, 4].

TRAPS, a dominantly inherited periodic fever [2–6], can be clinically distinguished from other hereditary periodic fevers by a number of characteristics: (1) recurrent attacks that often last >5 days and sometimes several weeks; (2) localized myalgia that is often associated with an overlying macular rash, which together display a centrifugal migratory pattern; (3) conjunctivitis and/or periorbital edema; (4) attenuation of symptoms with glucocorticoid but not with colchicine; (5) an autosomal-dominant mode of inheritance [21].

More than 100 different mutations have been reported for patients with TRAPS in INFEVERS, a mutational database accessible on the World Wide Web at http://fmf.igh.cnrs.fr/infevers. Of the seven mutations identified in the reported cases of Japanese TRAPS, five (C70S [14], T61I [15, 20], C70G [12], N101K [17], N25D [18]) have been reported only in Japan, while the remaining two (C30Y [16, 19], C30R [13]) have also been reported in Western countries. Since there are only 18 genetically confirmed TRAPS patients in Japan [12–20], further studies should be carried out to search for additional mutations among Japanese patients with TRAPS.

In our review of the nine case reports on Japanese TRAPS patients, eight index cases have family histories with periodic fever [12–16, 18–20], while there is only one sporadic case of TRAPS [17]. Ida and colleagues [15], who reported the T61I variant in TRAPS patients associated with systemic lupus erythematosus (SLE), reported that this variant was detected in five of 60 SLE patients (8.3 %) and five of the 120 healthy Japanese individuals in their study (4.2 %). They could not detect any significant difference in the proportion of this variant between the SLE patients and the healthy controls [15]. In addition, Horiuchi and colleagues [12], who reported on TRAPS patients with the C70G *TNFRSF1A* gene mutation, also reported that they identified the T61I variant in one of the 100 healthy Japanese volunteers in their study (1.0 %) [12]. In contrast, Aksentijevich and colleagues [9] also reported the presence of P46L and R92Q *TNFRSF1A* gene variants in TRAPS patients, which were also found in about 1 % of the U.S.

population. In particular, the R92Q mutation is supposed to be a low-penetration mutation that is associated with a milder disease course [11]. Assuming that these three substitutions confer susceptibility to autoinflammatory disease, the penetrance (i.e., the probability of having a disease if a person has a mutation) must be low because the frequency of TRAPS does not reach 1 % of the general population. The T61I, P46L, and R92Q mutations are generally considered to be low-penetrance *TNFRSF1A* gene variants because they are present in symptomatic patients as well as unaffected individuals [10].

The functional significance of T61I on *TNFRSF1A*, which augments TNF signaling, has been reported in Japan. The T61I variant has been associated with a defect in *TNFRSF1A* shedding in peripheral blood mononuclear cells [12]. However, other Japanese investigators did not find any effect on *TNFRSF1A* shedding in the monocytes collected from their patients carrying T61I [15]. In a specific cell population, such as lymphocytes, T61I may be related to the pathogenesis of TRAPS. As the T61I variation has not been reported in Caucasian patients with TRAPS, the clinical and functional importance of T61I variation needs to be clarified within the Japanese population.

There have been many sporadic cases of TRAPS in the absence of the *TNFRSF1A* gene mutation in both Japan [5] and Western countries [9]. However, we cannot eliminate the possibility sporadic cases without any gene mutation, which have been reported in the literature, had a novel gene mutation, as Nakamura and colleagues reported [18]. Since TRAPS was first proposed as a genetic diagnosis, only those patients with demonstrable TNF receptor mutations should have been included as being cases of TRAPS [2–6]. Further studies are recommended to argue this issue.

A review of the literature compiling 153 TRAPS patients from all over the world demonstrated that the most frequent symptom associated with fever is abdominal pain (77 %), which can occasionally lead to surgery in 33 % of TRAPS patients [10]. Other common clinical symptoms reported in this review are myalgia (63.5 %), rash (55.2 %), arthralgia (51 %), ocular involvement (48.8 %), and pleuritis (32 %) [10]. Arthralgia is more frequent than arthritis in TRAPS patients [4]. Chronic arthritis of the type seen in FMF has not been observed in TRAPS [4], and characteristic migratory myalgia and rashes, which distinguish TRAPS from FMF, typically occur as a localized area of cramping muscle pain with warmth and tenderness to palpation and an overlying erythematous, blanchable rash [4].

In our review of nine reports on Japanese TRAPS patients, the common clinical symptoms associated with fever were rash (erythema) (84.6 %), arthralgia (73.3 %), myalgia (54.5 %), and abdominal pain (50.0 %). Although half of the Japanese TRAPS patients suffered from abdominal pain, they had no history of abdominal surgery [15, 16, 19, 20]. In contrast, only small percentage of Japanese TRAPS patients suffered from chest pain (20.0 %), conjunctivitis (20.0 %), and headache (10.0 %). These findings suggest that the clinical symptoms of TRAPS may be milder in Japanese patients than in Caucasian ones, which is similar to the presentation of other inherited autoinflammatory syndromes, such as FMF [22] and HIDS [23]. The difference of the disease-causing mutations, the genetics or environmental background may be responsible for the discrepancies in the results. Further research is required before definitive conclusions can be drawn.

Since colchicine is ineffective in preventing the fever attacks, glucocorticoid can be used to treat the attacks of TRAPS, but patients will require escalating dosages over time [4–6]. Etanercept, an anti-TNF agent, is recommended for chronic therapy to prevent attacks [4–6]. Interleukin 1β blockade may be effective in cases resistant to anti-TNF therapy [24]. In our review of the Japanese literature, fever and rash improved after the administration of glucocorticoid in all patients [13–17, 19, 20], with the exception of the two patients who did not receive corticosteroid therapy [12, 18].

In our review of the Japanese literature, three of the nine index cases (30.0 %) and six of the 23 patients (26.1 %) had been misdiagnosed as having JIA or adult onset Still's disease prior to the diagnosis of TRAPS being established [13, 16, 19]. All of these patients had a family history of periodic fever [13, 16, 19]. Since glucocorticoid attenuates the clinical symptoms of TRAPS patients [21], patients misdiagnosed as having JIA or adult onset Still's disease may experience an improvement in their clinical symptoms with glucocorticoid therapy. There may be more TRAPS patients misdiagnosed as having JIA or adult Still's disease in Japan. Thus, an important issue should be whether Japanese patients diagnosed with JIA or adult onset Still's disease are actually TRAPS patients, especially if there is a family history of periodic fever.

There are a number of limitations to our review. First, the number of reported Japanese TRAPS patients is very small. In addition, insufficient clinical information was available for some of the family member patients of the index cases. Second, although all index cases are genetically confirmed TRAPS patients, some family members of index cases have been diagnosed as having TRAPS with clinical symptoms and this has been identified in their family history (i.e., kinship of index cases). Third, we cannot provide the answer to whether the differences between Japanese and Western TRAPS is dependent on the difference in the disease-causing mutations, genetics, or environmental background. Further studies are recommended to provide this answer.

The major strength of this review is that it demonstrates that clinical symptoms are milder in Japanese TRAPS patients than in TRAPS patients in Western countries. Compared with their counterparts, Japanese patients are less likely to suffer from severe abdominal pain, and none of the patients in this review had a medical history of abdominal surgery.

In conclusion, our review of the Japanese literature possibly suggests that the clinical features of Japanese patients with TRAPS are milder than those of TRAPS patients in Western countries. We have launched a national survey of TRAPS patients in Japan [6] and are investigating the modified diagnostic criteria for Japanese patients with TRAPS.

Acknowledgments This work was supported in part by a Grant for Research on Intractable Disease from Japanese Ministry of Health, Labour and Welfare (Chief: Takahiko Horiuchi).

Conflict of interest None.

References

1. Williamson LM, Hull D, Mehta R, Reeves WG, Robinson BH, Toghill PJ. Familial Hibernian fever. Q J Med. 1982;51:469–80.
2. McDermott MF, Aksentijevich I, Galon J, McDermott EM, Ogunkolade BW, Centola M, et al. Germline mutations in the extracellular domains of the 55 kDa TNF receptor, TNFR1, define a family of dominantly inherited autoinflammatory syndromes. Cell. 1999;97:133–44.
3. Hull KH, Drewe E, Aksentijevich I, Singh HK, Wong K, McDermott EM, et al. The TNF receptor-associated periodic syndrome (TRAPS), emerging concepts of an autoinflammatory disorder. Medicine. 2002;81:349–68.
4. Colburn N, Kastner D. Hereditary periodic fever. In: Hochberg MC, Silman AJ, Smolen JS, Weinbalatt ME, Weisman MH, editors. Rheumatology. 4th ed. Philadelphia: Elsevier; 2008. p. 1619–40.
5. Ida H, Eguchi K. TNF receptor-associated periodic syndrome (TRAPS) in Japan: clinical characterization, pathogenesis, diagnostic criteria, and treatment (in Japanese). Jpn J Clin Immunol. 2007;30(2):90–100.
6. Tsukamoto H, Ueda N, Horiuchi T. Progress in classification and treatment for TNF receptor-associated periodic syndrome (in Japanese). Jpn J Clin Immunol. 2011;34(5):361–8.
7. Lobito AA, Kimberley FC, Muppidi JR, Komorow H, Jackson AH, Hull KM, et al. Abnormal disulfide-linked oligomerization results in ER retention and altered signaling by TNFR1 mutations in TNFR1-associated periodic fever syndrome (TRAPS). Blood. 2006;108:1320–7.
8. Simon A, Park H, Maddipati R, Lobito AA, Bulua AC, Jackson AJ, et al. Concerted action of wild-type and mutant TNF receptors enhances inflammation in TNF receptor 1-associated periodic fever syndrome. Proc Natl Acad Sci USA. 2010;107(21):9801–6.
9. Aksentijevich I, Galon J, Soares M, Mansfield E, Hull K, Oh HH, et al. The tumor-necrosis-factor receptor-associated periodic syndrome: new mutations in TNFRSF1A, ancestral origins, genotype–phenotype studies, and evidence for further genetic heterogeneity of periodic fevers. Am J Hum Genet. 2001;69(2):301–14.
10. Stojanov S, McDermott MF. The tumor necrosis factor receptor-associated periodic syndrome: current concepts. Expert Rev Mol Med. 2005;7:1–18.
11. Pelagatti MA, Meini A, Caorsi R, Cattalini M, Federici S, Zulian F, et al. Long-term clinical profile of children with the low-penetrance R92Q mutation of the TNFRSF1A gene. Arthritis Rheum. 2011;63(4):1141–50.
12. Horiuchi T, Tsukamoto H, Mitoma H, Miyagawa H, Tamimoto Y, Yoshizawa S, et al. Novel mutations in TNFRSF1A in patients with typical tumor necrosis factor receptor-associated periodic syndrome and with systemic lupus erythematosus in Japanese. Int J Mol Med. 2004;14:813–8.
13. Takagi K, Kawaguchi Y, Fujikawa S, Otani T, Sugiura T, Hara M. Tumor necrosis factor receptor-associated periodic syndrome with a C30R mutation in a Japanese Family. Mod Rheumatol. 2007;17:265–6.
14. Kusuhara K, Nomura A, Nakao F, Hara T. Tumor necrosis factor receptor-associated periodic syndrome with a novel mutation in the TNFRSF1A gene in a Japanese family. Eur J Pediatr. 2004;163:30–2.
15. Ida H, Kawasaki E, Miyashita T, Tanaka F, Kamachi M, Izumi Y, et al. A novel mutation (T61I) in the gene encoding tumor necrosis factor receptor superfamily 1A (TNFRSF1A) in a Japanese patients with tumor necrosis factor-associated with periodic syndrome (TRAPS) associated with systemic lupus erythematosus. Rheumatology (Oxford). 2004;43:1292–9.
16. Manki A, Nishikomori R, Nakata-Hizume M, Kunitomi T, Takei S, Urakami T, et al. Tumor necrosis factor receptor-associated periodic syndrome mimicking systemic juvenile idiopathic arthritis. Allergol Int. 2006;55:337–41.
17. Nakamura M, Kobashi M, Tokura Y. A novel missense mutation in tumor necrosis factor receptor superfamily 1A (TNFRSF1A) gene found in tumor necrosis factor receptor-associated periodic syndrome (TRAPS) manifesting adult-onset Still disease-like skin eruptions: report of a case and review of literature. Br J Dermatol. 2009;161:968–70.
18. Nakamura M, Tokura Y. A novel missense mutation in tumor necrosis factor receptor superfamily 1A (TNFRSF1A) gene found in tumor necrosis factor receptor-associated periodic syndrome (TRAPS) with high serum interleukin (IL)-22. Eur J Dermatol. 2010;20(4):508–9.
19. Kai M, Tamaki S, Nishikomori R, Takaoka Y, Ohara O, Oshima K. A case of TNF receptor-associated periodic syndrome (in Japanese). Ryumachika (Rheumatology). 2011;45(4):456–60.
20. Ohmori S, Hino R, Nakamura M, Tokura Y. Heparin serves as a natural stimulant of the inflammasome and exacerbates the symptoms of tumor necrosis factor receptor-associated periodic syndrome (TRAPS). J Dermatol Sci. 2012;66:82–4.
21. Hull KM, Wong K, Wood GM, Chu WS, Kastner DL. Monocytic fasciitis: a newly recognized clinical feature of tumor necrosis factor receptor dysfunction. Arthritis Rheum. 2002;46(8):2189–94.
22. Tsuchiya-Suzuki A, Yamazaki M, Nakamura A, Yamazaki K, Agematsu K, Matsuda M, et al. Clinical and genetic features of familial Mediterranean fever in Japan. J Rheumatol. 2009;36(8):1671–6.
23. Sasaki H, Heike T. Hyperimmunoglobulinemia D and periodic fever syndrome (in Japanese). Jpn J Clin Immunol. 2011;34(5):382–7.
24. Sacré K, Brihaye B, Lidove O, Papo T, Pocidalo MA, Cuisset L, et al. Dramatic improvement following interleukin 1beta blockade in tumor necrosis factor receptor-1-associated syndrome (TRAPS) resistant to anti-TNF-alpha therapy. J Rheumatol. 2008;35(2):357–8.

Tanaka et al. Arthritis Research & Therapy 2012, 14:R244
http://arthritis-research.com/content/14/6/R244

arthritis research&therapy

RESEARCH ARTICLE Open Access

Serum progranulin levels are elevated in patients with systemic lupus erythematosus, reflecting disease activity

Atsushi Tanaka[1], Hiroshi Tsukamoto[1*], Hiroki Mitoma[2,3], Chikako Kiyohara[4], Naoyasu Ueda[1], Masahiro Ayano[1], Shun-ichiro Ohta[1], Yasushi Inoue[1], Yojirou Arinobu[5], Hiroaki Niiro[1], Takahiko Horiuchi[1] and Koichi Akashi[1*]

Abstract

Introduction: Progranulin (PGRN) is the precursor of granulin (GRN), a soluble cofactor for toll-like receptor 9 (TLR9) signaling evoked by oligonucleotide (CpG)-DNA. Because TLR9 signaling plays an important role in systemic lupus erythematosus (SLE), we investigated whether PGRN is involved in the pathogenesis of SLE.

Methods: We measured concentrations of serum PGRN and interleukin-6 (IL-6) with enzyme-linked immunosorbent assay (ELISA) in patients with SLE (n = 68) and in healthy controls (n = 60). We assessed the correlation between the serum PGRN levels and established disease-activity indexes. The sera from the patients with high PGRN titers (>80 ng/ml) at the initial evaluation were reevaluated after the disease was ameliorated by treatment. We also measured the IL-6 concentration secreted by peripheral blood mononuclear cells (PBMCs) incubated with (a) oligonucleotide (CpG-B) in the presence or absence of recombinant human PGRN (rhPGRN); and (b) lupus sera in the presence or absence of a neutralizing anti-PGRN antibody.

Results: Serum PGRN levels were significantly higher in SLE patients than healthy controls. Their levels were significantly associated with activity of clinical symptoms. They also significantly correlated with values of clinical parameters, including the SLE Disease Activity Index and anti-double-stranded DNA antibody titers, and inversely with CH50, C3, and C4 levels. Moreover, serum PGRN levels significantly decreased after successful treatment of SLE. The rhPGRN significantly upregulated the production of IL-6 by PBMCs stimulated with CpG-B. Patients' sera stimulated production of IL-6 from PBMCs, which was significantly impaired by neutralization of PGRN. The serum PGRN levels significantly correlated with the serum IL-6 levels.

Conclusions: Serum PGRN could be a useful biomarker for disease activity of SLE. PGRN may be involved in the pathogenesis of SLE partly by enhancing the TLR9 signaling.

Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune and inflammatory disease characterized by the polyclonal activation of T and B lymphocytes, production of autoantibodies, and formation of immune complexes that result in tissue and organ damage [1,2].

Toll-like receptor (TLR) signaling contributes to innate and adaptive immune responses [3]. TLR9 is a receptor for microbial CpG-DNA [4] and is expressed in plasmacytoid dendritic cells (pDCs), macrophages, and B-lymphocytes [5]. TLR9 recognizes unmethylated CpG oligonucleotides (CpG-ODNs), which are generally not present in mammalian cells [6]. However, in SLE, nucleic acid-containing autoantigens can be generated from apoptotic or necrotic cells [7] because of increased apoptosis, reduced clearance of apoptotic cells [8], and decreased methylation of DNA [9]. Patients with active SLE have increased TLR9 expression in peripheral blood memory and plasma B lymphocytes [10], and TLR9 signaling controls anti-DNA autoantibody production from these B cells in murine [11] and human lupus [12]. A genetic variation of TLR9 is associated with an increased

* Correspondence: tsukamot@intmed1.med.kyushu-u.ac.jp; akashi@med.kyushu-u.ac.jp
[1]Department of Medicine and Biosystemic Science, Kyushu University, Graduate School of Medical Sciences, Fukuoka 812-8582, Japan
Full list of author information is available at the end of the article

risk of SLE [13]. These lines of evidence suggest that TLR9 signaling may play an important role in the pathogenesis of SLE.

Progranulin (PGRN; GenBank: NC_000017) is an extracellular glycoprotein, containing seven and a half repeats of cysteine-rich motifs. PGRN is proteolytically cleaved by extracellular proteases, such as proteinase 3 (PR3) and elastase, into granulin (GRN) [14], which ranges from 6 to 25 kDa. PGRN is abundantly expressed in rapidly cycling epithelial cells, leukocytes, chondrocytes, and neurons [15], and its expression level is at steady state [16]. PGRN plays a critical role in early embryogenesis [16], wound healing [17], maintenance of neuronal survival [18], and tumorigenesis [15]. Recent mouse studies show that mice unable to convert PGRN into GRN because of lack of both elastase and PR3 cannot show inflammation in response to injection of immune complexes [19]. These data indicate that PGRN is rapidly cleaved into GRNs in tissues by elastase to enhance inflammation. Moreover, GRN acts as a soluble cofactor for TLR9 signaling by binding to both CpG-ODNs and TLR9, thereby acting as a cross-linker for their interaction. GRN also promotes the delivery of CpG-ODNs to the endolysosomal compartments where TLR9 is localized [20].

Here, we show that serum PGRN levels are significantly elevated in SLE patients in parallel with disease activities and that PGRN may have a role in the pathogenesis of SLE partly by enhancing the TLR9 signaling and IL-6 production.

Materials and methods

Patients

We performed a cross-sectional study of patients who were treated for SLE at the Kyushu University hospital between the years of 2005 and 2011. In total, 68 Japanese patients with SLE were enrolled, and sera were obtained from these patients. All of the patients fulfilled at least four of the American College of Rheumatology (ACR) revised criteria for SLE. SLE disease activity was measured by using the SLE Disease Activity Index (SLEDAI) [21]. Active SLE was defined as a SLEDAI score ≥6 [21]. We excluded other autoimmune and infectious diseases. Each patient completed a standardized medical history, including drug use, and wās given a physical examination. Serologic profiling of each patient was performed by using the standard immunoassays described later. The serum samples from the active SLE patients were acquired before the initiation or reinforcement of treatment, and the samples from the inactive SLE patients were acquired during regular hospital visits and then stored at -20°C.

The active SLE patients were treated with corticosteroids or immunosuppressive drugs after the completion of these evaluations. The sera obtained from the patients with high PGRN titers (>80 ng/ml) at the initial evaluation were reevaluated after the disease was ameliorated by treatment (n = 15). Control sera were obtained from healthy staff members (n = 60) in our hospital. This study was approved by the ethics committee of our institution, and the principles of the Helsinki Declaration were followed throughout the study. Informed consent was obtained from all participants.

Data collection

The information obtained from the medical records of the patients included demographic data, such as age, sex, clinical manifestations of SLE, and laboratory values. Each SLE-related feature except anemia was defined according to the SLEDAI. Clinical features checked were malar rash/discoid rash, alopecia, oral or nasal ulcers, serositis, arthritis, active nephritis, CNS (central nervous system) lupus, vasculitis, fever >38°C, thrombocytopenia, leukopenia, and anemia. For example, thrombocytopenia was defined as a decrease in the number of platelets to <100,000/mm^3. Leukopenia was defined as a decrease in the number of white blood cells to <3,000/mm^3. Anemia was defined as a decrease in the concentration of hemoglobin to <10.0 g/dl for any cause. The data for the serum anti-Smith (anti-Sm), anti-ribonucleoprotein (anti-nRNP), anti-Ro/SS-A (anti-Ro), and anti-cardiolipin (anti-CL) antibodies were collected only from active SLE patients in whom these autoantibodies were measured (anti-Sm, n = 42; anti-nRNP, n = 35; anti-Ro, n = 39; and anti-CL, n = 44).

The measurement of complement and autoantibody levels

The serum C3 and C4 levels were measured with turbidimetric immunoassay (Nittobo, Tokyo, Japan). The serum CH50 levels were measured with liposome immunosorbent assay (Wako, Tokyo, Japan). The serum anti-dsDNA, anti-Sm, anti-nRNP, and anti-Ro antibody levels were measured by using fluorescence-enzyme immunoassay (Phadia, Tokyo, Japan). The serum anti-CL antibody levels were measured with ELISA (MBL, Nagano, Japan).

Immunoprecipitation

A primary antibody for PGRN (anti-PGRN Ab; R&D Systems, Minneapolis, MN, USA) or an isotype control antibody (control Ab; R&D Systems) was added to protein G-Sepharose (Pierce, Rockford, IL, USA) in phosphate-buffered saline (PBS) with 0.5% Triton-X and incubated on a shaker for 4 hours at 4°C. Recombinant human PGRN (rhPGRN; Cedarlane, Burlington, NC, USA) was added to the anti-PGRN Ab- or control Ab-protein G-Sepharose complexes and incubated on a

shaker for 4 hours at 4°C. After washing the complexes, the proteins were eluted by boiling in sample buffer (0.125 *M* Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 3.1% dithiothreitol, and bromophenol blue). Proteins were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a nitrocellulose membrane, blocked with 5% skim milk in PBS with 0.1% Tween-20 for 1 hour, and probed with another anti-PGRN Ab (Epitomics, Burlingame, CA, USA) overnight at 4°C. The membranes were washed and incubated with horseradish peroxidase-conjugated streptavidin (Pierce) for 1 hour. The immunoblots were visualized with ECL detection reagent (Pierce).

IL-6 induction

Peripheral blood mononuclear cells (PBMCs; 4×10^5 cells) from healthy control individuals were cultured with 250 ng/ml rhPGRN in serum-free medium (Invitrogen, Grand Island, NY, USA) for 2 hours to let PGRN partially convert into GRNs and were then stimulated with 10 n*M* CpG-B, 100 ng/ml poly (I:C) (agonist for TLR3) (InvivoGen, San Diego, CA, USA), 60 pg/ml LPS (agonist for TLR4) (Sigma-Aldrich, Saint Louis, MO, USA), or 1 μg/ml imiquimod (R837; agonist for TLR7) (InvivoGen). The PBMCs were also stimulated with 10% serum from SLE patients (n = 4) in the presence of anti-PGRN Ab or control Ab. In some experiments, lupus sera (n = 3) were treated for 1 hour with 6,000 U/ml DNase I (Roche, Penzberg, Germany) before stimulation. Triplicate cultures were grown in 96-well plates (Becton Dickinson, Franklin Lakes, NJ, USA) at a final volume of 200 μl/well. After 24 hours, the amount of IL-6 in the supernatant was measured.

Measurement of PGRN, IL-6, IL-10, and immune complexes

The serum PGRN levels, serum and cell-culture supernatant IL-6 and IL-10 levels were determined by using ELISA kits (R&D Systems) according to the manufacturer's protocol. In brief, for PGRN, calibrators, control sera, and patients' sera (stored samples) were diluted and incubated with a mouse monoclonal antibody against PGRN, adsorbed onto the microtiter plate wells. After washing, a mouse monoclonal antibody against PGRN conjugated to horseradish peroxidase was added, followed by a second washing step and the addition of tetramethylbenzidine substrate. The intensity of the blue color developed was in proportion to the amount of PGRN bound in the initial step. The reaction was terminated by the addition of 2N sulfuric acid. The absorbance was measured in a microtiter plate reader (ThermoFisher Scientific, Waltham, MA, USA) and converted into nanograms per milliliter by plotting the values against the PGRN titer of the calibrators/standards given by the manufacturer. The assay range was 1.56 to 100 ng/ml.

Serum levels of immune complexes were measured with C1q solid-phase enzyme immunoassay according to the manufacturer's protocol (TFB, Tokyo, Japan).

Statistical analysis

The differences between two groups were analyzed by using the Student t test. If there were a significant difference between the variances of the two samples, the t test corrected for unequal variances (the Welch t test) was applied. The Dunnett test is used for multiple comparisons with a control group. The relations between PGRN levels and other continuous variables were analyzed by using the Spearman rank correlation. The PGRN levels before and after treatment were compared by using a paired t test. The SLE patients were divided into three groups based on tertile distribution of the number of clinical features (0, 1 to 2, and 3 to 8). Linear trend across the tertiles was assessed by using ordinal variables coded 1 to 3. Among three groups, P values were calculated by analysis of variance and were adjusted by use of the Bonferroni correction. P values <0.05 were considered significant. All tests were two-tailed. All analyses were performed by using JMP statistical software (SAS Institute).

Results

The serum PGRN levels were elevated in patients with SLE

Of the 68 patients with SLE enrolled in the present study, 58 were women, and 10 were men (active, n = 46; inactive, n = 22). The patients ranged in age from 17 to 76 years (median age, 37 years). Meanwhile, in healthy controls, 51 were women, and nine were men. They ranged in age from 20 to 59 years (median age 32 years). No significant differences were found between patients with SLE and controls in terms of age and gender.

To investigate the role of PGRN and/or GRN in the pathogenesis of SLE, we first compared serum PGRN levels between 68 patients with SLE and 60 healthy controls by using ELISA (Figure 1). Serum PGRN levels in controls were always within the range of 35 to 70 ng/ml and distributed normally. Serum PGRN levels in patients with SLE (mean, 87.6 ng/ml) were significantly and markedly higher than those in healthy controls (49.3 ng/ml; $P < 0.0001$). It is of note that serum PGRN levels >100 ng/ml were found in 19 of the 68 SLE patients (27.9%), but never in healthy controls.

The serum PGRN levels in SLE patients correlated with disease activities

We next tested whether serum PGRN levels correlate with serologic parameters for disease activity of SLE.

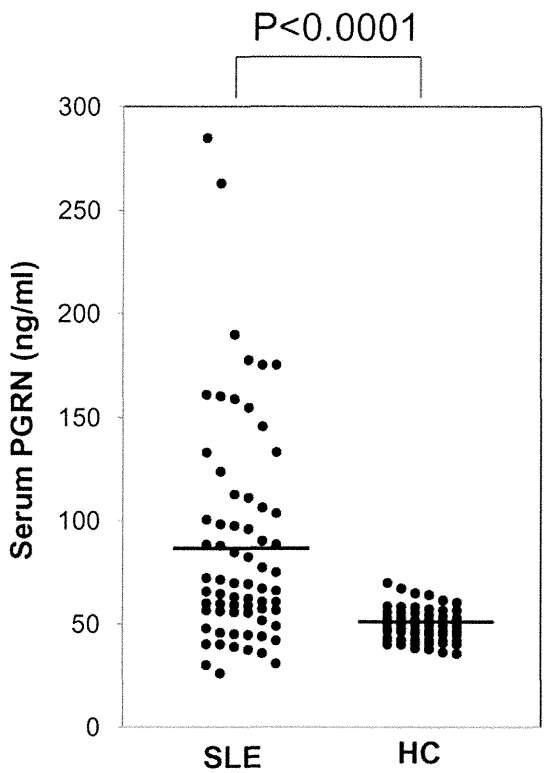

Figure 1 The serum progranulin (PGRN) levels are elevated in patients with systemic lupus erythematosus (SLE). Serum PGRN levels in 68 patients with SLE and 60 healthy controls (HCs) are shown, as measured with enzyme-linked immunosorbent assay (ELISA). The serum PGRN levels in SLE patients were significantly higher than those in HCs.

Serum PGRN levels showed a significantly positive correlation with SLEDAI (rs = 0.53; P = 0.0003; Figure 2A). They also significantly correlated with the titer of anti-dsDNA antibodies (rs = 0.45; P = 0.0023; Figure 2B) and inversely with serum levels of C3 (rs = -0.41; P = 0.01; Figure 2C), C4 (rs = -0.37; P = 0.002; Figure 2D), and CH50 (rs = -0.47; P = 0.0035; Figure 2E). Moreover, in 15 lupus patients who had high serum PGRN levels (>80 ng/ml) at the initial evaluation, after ameliorating the disease by treatment, serum PGRN levels were significantly decreased (P = 0.0001; Figure 3). Serum PGRN levels reflected disease activities of SLE.

The high serum PGRN levels were associated with the accumulation of the clinical features of SLE

We next investigated the relation between SLE-related clinical features and serum PGRN levels. Patients who had positive levels of anti-nRNP antibodies showed significantly elevated levels of serum PGRN (P = 0.002; Table 1). The maximum number of coexisting clinical features was eight (n = 1). When classified by the number of clinical features, serum PGRN levels elevated significantly, with increasing tertile of clinical feature (Table 1, P for trend = 0.005). Thus, the high serum PGRN levels were associated with the accumulation of the clinical features of SLE.

PGRN is involved in the production of IL-6 from human PBMCs

GRN enhances the CpG-induced TLR9 signaling (not TLR 3, 4, or 7 signaling) and promotes the production of IL-6, an inflammatory cytokine related to the pathogenesis of SLE, in murine macrophages [20]. Accordingly, we tested whether PGRN is involved in secretion of IL-6 via TLR9 signaling by human PBMCs. In the lupus model mouse, TLR 3, 4, 7, and 9 signaling are reported to be involved in the pathogenesis of lupus [22-24]. PBMCs from healthy controls were incubated with 10 nM (sub-optimal concentration in our case; data not shown) CpG-B, 60 pg/ml LPS, 100 ng/ml poly (I:C), or 1 μg/ml imiquimod for 24 hours with or without 250 ng/ml rhPGRN. This rhPGRN concentration is slightly lower than the PGRN concentration we found in patients' sera (Figure 1).

The addition of rhPGRN to the culture media significantly stimulated PBMCs to produce IL-6 in the presence of CpG-B (P < 0.05; Figure 4A). However, IL-6 production induced by rhPGRN was not augmented in the presence of poly (I:C), LPS, or imiquimod (Figure 4A), as previously reported [20]. Lupus serum contains immune complexes and is able to induce the production of IL-6 and IL-10 from PBMCs [25,26]. Actually, immune complexes were present in patients' sera we used (Patients 1 through 7, median, 6.1 μg/ml; range, 2.6 to 9.7 μg/ml). Those levels in healthy controls (n = 7) were less than 1.5 μg/ml. Consistent with the previous reports, by incubation with patients' sera, PBMCs from healthy controls were stimulated to produce considerable amounts of IL-6 and IL-10 (Figure 4B, and data not shown).

We then tested whether this stimulation of IL-6 and IL-10 production is dependent on the PGRN contained in patients' sera. To this end, we used an anti-PGRN antibody that can bind to PGRN demonstrated by immunoprecipitation and immunoblot analyses (Figure 4B, bottom). We incubated PBMCs with 10% patients' sera supplemented with the anti-PGRN Ab or with the control Ab for 24 hours. The neutralization of PGRN significantly impaired the effect of patients' sera on IL-6 production from normal PBMCs (P < 0.05) in all four cases tested (Figure 4B, top; reduction range, 42.8% to 84%). Although IL-10 production tended to be reduced by the neutralization of PGRN, the level of reduction was not significant (data not shown).

We then tested whether TLR9 signaling is involved in this reduction of IL-6 production by neutralizing the

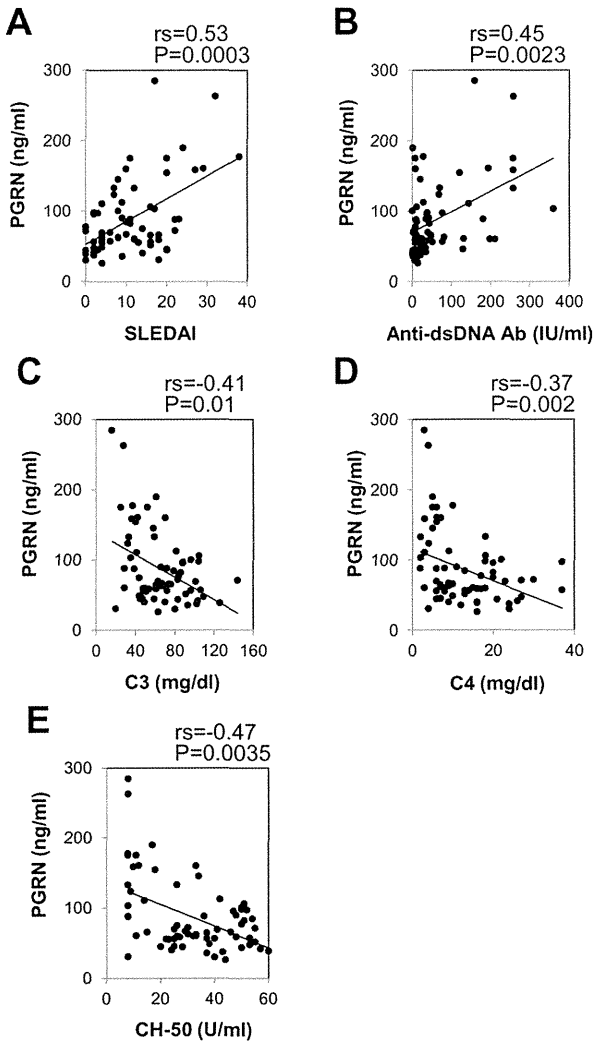

Figure 2 The serum progranulin (PGRN) levels in systemic lupus erythematosus (SLE) patients correlate with disease activities. Correlations are shown between the serum PGRN levels and the SLEDAI scores, the titers of **(A)** anti-dsDNA antibody (anti-dsDNA Ab) **(B)**, the serum levels of C3 **(C)**, C4 **(D)**, and CH50 **(E)**, as measured in 68 patients with SLE. For all comparisons, significant positive or negative correlations were observed.

PGRN contained in patients' sera. To this end, we treated lupus sera (n = 3) with 6,000 U/ml DNase I for 1 hour before stimulation. The degradation of DNA significantly impaired the effect of patients' sera on IL-6 production from normal PBMCs. The degree of suppression was almost as same as that caused by the neutralization of PGRN ($P < 0.05$; Figure 4C).

We further examined the correlation between concentrations of PGRN and IL-6 in patients' sera. As expected, serum PGRN levels showed a significantly positive correlation with serum IL-6 levels (rs = 0.47; $P < 0.0001$; Figure 4D). These results suggest that serum PGRN and/or GRN is an important cofactor in the production of IL-6 in SLE patients.

Discussion

This is the first study to show that PGRN levels are significantly elevated in sera of SLE patients, and their concentrations were correlated with disease activity and serum levels of IL-6. Thus, PGRN and/or GRN in patients' sera play an important role in the production of IL-6 from PBMCs.

PGRN is produced by various types of cells [15], but the mechanism of the expression control of PGRN is not well understood. In humans, the elevation of PGRN is observed in local inflammatory tissues, such as in brains in patients with active multiple sclerosis [27] and in the synovium of rheumatoid arthritis (RA) patients [28]. We measured concentrations of serum PGRN in patients

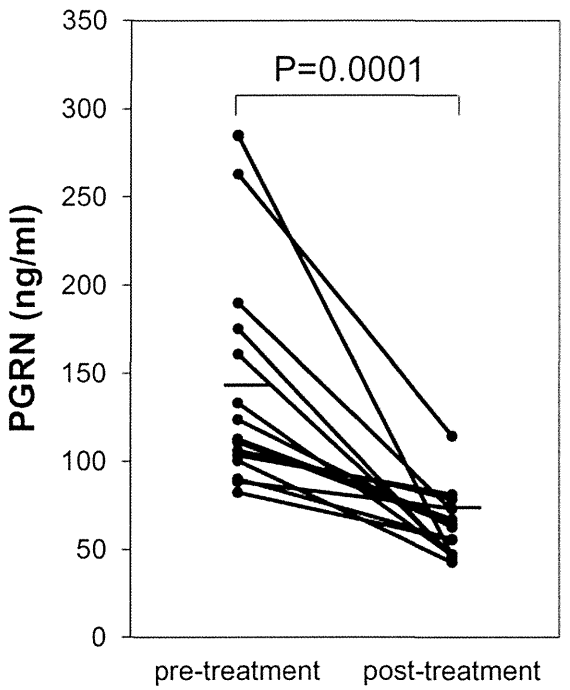

Figure 3 Serum progranulin (PGRN) levels are significantly decreased after ameliorating the disease with treatment. Serum PGRN levels before and after treatment in 15 patients with active systemic lupus erythematosus (SLE) who had serum PGRN levels >80 ng/ml at the initial evaluation. The serum PGRN levels decreased significantly with the clinical amelioration of the disease from the median SLEDAI score of 11 to 4 after treatment.

with RA (n = 33). In our data, although sera from RA patients contained significantly elevated PGRN (mean, 54.7 ng/ml) compared with healthy controls (P = 0.0138), SLE patients' sera had much higher levels of PGRN.

PGRN is converted to GRN by the elastase produced by leukocytes and other cells *in vivo* and *in vitro* [29]. In contrast to that with PGRN, recent mouse studies have shown that GRN possess inflammatory functions [19,30,31]. Because we cannot measure serum GRNs, it is difficult to investigate the function of GRN in human studies.

Our data also show that rhPGRN enhances IL-6 production from human PBMCs in the presence of TLR9 signaling triggered by CpG, but does not enhance TLR3, 4, or 7 signaling (Figure 4A). In addition, patients' sera stimulated PBMCs to produce IL-6, whereas neutralization of PGRN in patients' sera dramatically attenuated such IL-6 production from PBMCs, almost the same as the degradation of DNA in patients' sera (Figure 4B and 4C). IL-6 production in lupus is reported to mediate by immune complexes that transmit signals through TLR7 and 9 [22,32-34] and Fcγ receptor [26]. Our data suggest that DNA-containing immune complexes in patients' sera stimulate IL-6 production from healthy PBMCs, mainly via TLR9 signaling, and that serum PGRN and/or GRN may act as an important cofactor of TLR9 signaling. Conversely, because the augmentation of IL-6 production induced by CpG only in the presence of rhPGRN was limited, it is suggested that cofactors other than PGRN may be required to form immune complexes that effectively stimulate TLR9 signaling.

In our analysis, serum PGRN levels were significantly correlated with serum titers of anti-dsDNA and anti-nRNP antibodies. IL-6 plays a role in patients with SLE, especially on the production of immunoglobulin [35-39]. In addition, lupus B lymphocytes are hypersensitive to IL-6 [40,41]. Thus, induction of IL-6 by PGRN and/or GRN could be a mechanism by which PGRN is correlated with the production of these autoantibodies.

Table 1 Associations between systemic lupus erythematosus (SLE)-related serologic and clinical features and titer of progranulin (PGRN)

Feature	Number	Mean PGRN (95% confidence interval)		P
		Positive[a]	Negative[a]	
Serologic feature[b]	Positive/negative			
Anti-Sm	25/17	111.8 (93.4-130.1)	94.1 (56.5-131.7)	0.381[c]
Anti-nRNP	23/12	128.9 (102.7-155.0)	74.9 (53.5-96.3)	0.002[c]
Anti-Ro	26/13	102.7 (79.6-125.8)	105.6 (67.6-143.6)	0.888
Anti-CL	20/24	112.4 (84.0-140.8)	87.4 (65.2-109.6)	0.150
Clinical features[d]				
0	22	51.3 (34.5-68.1)		<0.001[ef]
1-2	31	91.8 (81.1-102.5)		<0.001[e]
3-8	15	132.3 (113.1-151.5)		0.005[g]

anti-Sm, anti-Smith antibody; anti-nRNP, anti-ribonucleoprotein antibody; anti-Ro, anti-Ro/SS-A antibody; anti-CL, anti-cardiolipin antibody. [a]SLE patients with (present) or without (absent) the clinical features of SLE. [b]Several observations with missing values. [c]Welch t test. [d]Number of positive clinical features including malar rash/discoid rash, alopecia, oral or nasal ulcers, serositis, arthritis, active nephritis, CNS (central nervous system) lupus, vasculitis, temperature >38°C, thrombocytopenia, leukopenia, anemia. [e]As compared with three to eight clinical features (one-way analysis of variance followed by the Bonferroni test). [f]As compared with one to two clinical features (one-way analysis of variance followed by the Bonferroni test). [g]P for trend.

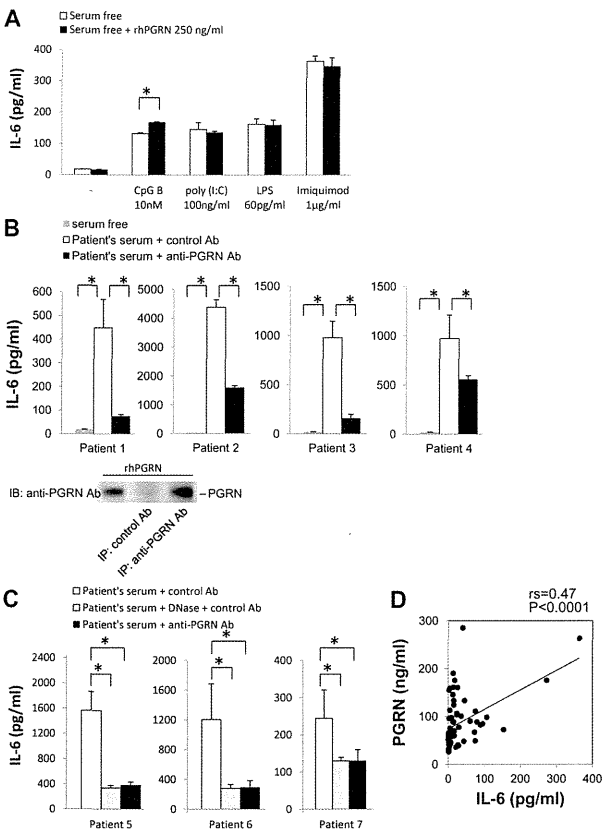

Figure 4 Progranulin (PGRN) and/or its fragments GRNs augment the IL-6 production by human peripheral blood mononuclear cells (PBMCs) via TLR9 signaling. (A) PBMCs from healthy control individuals were incubated for 24 hours in serum-free medium with or without 10 n*M* CpG-B, 100 ng/ml poly (I:C), 60 pg/ml LPS, or 1 μg/ml imiquimod and purified PGRN (250 ng/ml). The supernatant IL-6 levels were measured with ELISA. The data are representative of three independent experiments (average and SEM). **(B, C)** The binding of anti-PGRN Ab to PGRN was verified by immunoprecipitation and immunoblot with anti-PGRN Ab. In C, lupus sera were partly treated for 1 hour with 6,000 U/ml DNase I before stimulation. The PBMCs were then incubated in serum-free medium or were stimulated with 10% serum from systemic lupus erythematosus (SLE) patients in the presence of anti-PGRN Ab or control Ab for 24 hours. The supernatant IL-6 levels were measured with ELISA. **(D)** The serum PGRN levels correlated significantly with the serum IL-6 levels in SLE patients. *$P < 0.05$.

Interestingly, anti-Ro antibody, which is strongly associated with increased interferon-pathway activation in SLE [42], was not associated with serum PGRN levels, indicating that PGRN may be involved in the pathogenesis of SLE, independent of the interferon pathway. In addition, serum PGRN levels were significantly elevated with increasing clinical features. Individually, serum PGRN levels were significantly higher in the SLE patients who developed dominant clinical features, namely anemia (P = 0.019, data not shown) and arthritis (P = 0.002, data not shown). The elevated IL-6 and enhanced inflammation could accelerate these clinical features. IL-6 impairs iron utilization, leading to anemia of chronic disease. In addition, IL-6 promotes arthritis in patients with SLE [37]. Thus, the induction of IL-6 via PGRN is a possible explanation for the fact that PGRN levels are correlated with anemia and arthritis.

We must acknowledge some limitations of this study. The sample size of our study was small. Statistical tests usually require a larger sample size to justify that the effect did not happen by chance alone. Moreover, because of its cross-sectional design, it is difficult to establish the exact and definite causal relations, except the association between PGRN and development of SLE from the collected data.

In conclusion, this pilot study demonstrated that the serum PGRN levels were elevated in patients with SLE and were associated with the systemic disease activity. PGRN could be a useful biomarker for disease activity and may be involved in the pathogenesis of SLE, partly by enhancing the TLR9 signaling. Further studies are required to reveal more-precise mechanisms of PGRN and GRN in human autoimmune diseases and in host defense against microbes.

Conclusions

The present study demonstrated that the serum levels of PGRN, a fragment of which (GRN) is a soluble cofactor for TLR9 signaling, were elevated in patients with SLE and were associated with the systemic disease activity. PGRN may have a role in the pathogenesis of SLE, partly by affecting TLR9 signaling, and could be a useful biomarker for disease activity. These findings provide new insights into the pathogenesis as well as the therapy of SLE, and shed new light on the dysregulation of the immune system in autoimmune diseases.

Abbreviations

ACR: American College of Rheumatology; anti-CL: anti-cardiolipin; anti-nRNP: anti-ribonucleoprotein; anti-PGRN Ab: antibody for PGRN; anti-Ro: anti-Ro/SS-A; control Ab: isotype control antibody; CpG-ODN: CpG oligonucleotide; ELISA: enzyme-linked immunosorbent assay; GRN: granulin; PBMCs: peripheral blood mononuclear cells; PBS: phosphate-buffered saline; pDC: plasmacytoid dendritic cell; PGRN: progranulin; PR3: proteinase 3; RA: rheumatoid arthritis; rhPGRN: recombinant human PGRN; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SLE: systemic lupus erythematosus; SLEDAI: SLE Disease Activity Index; anti-Sm: anti-Smith; TLR: toll-like receptor.

Acknowledgements

This work was supported in part by grants from the Ministry of Health, Labor and Welfare (H22-Immunity-Ippan-008); and from the Ministry of Education, Science, Sports, and Culture of Japan (21591266). We thank nature publishing group language editing for proof reading this article. We are grateful to the patients and volunteers for their participation in this study and thank for all the rheumatologists in the Department of Medicine and Biosystemic Science of Kyushu University, who participated in this study.

Author details

[1]Department of Medicine and Biosystemic Science, Kyushu University, Graduate School of Medical Sciences, Fukuoka 812-8582, Japan. [2]Department of Internal Medicine, Saga University, 5-1-1 Nabeshima, Saga, 849-8501, Japan. [3]Research Fellowship Division Japan Society for the Promotion of Science, Sumitomo-Ichibancho FS Bldg., 8 Ichibancho, Chiyoda-ku, Tokyo 102-8472, Japan. [4]Department of Preventive Medicine, Kyushu University Graduate School of Medical Sciences, Fukuoka 812-8582, Japan. [5]Center for Cellular and Molecular Medicine, Kyushu University Hospital, Fukuoka 812-8582, Japan.

Authors' contributions

AT performed the experiments, the statistical analysis, and prepared the manuscript. HT, HM, TH and KA designed the study and helped to draft the manuscript. CK helped the statistical analysis and the manuscript edit. NU, MA, and SO assisted in conducting the experiments. YI, YA and HN contributed to data analysis and interpretation. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 29 March 2012 Revised: 15 October 2012
Accepted: 9 November 2012 Published: 11 November 2012

References

1. Kotzin BL: **Systemic lupus erythematosus.** *Cell* 1996, **85**:303-306.
2. Mills JA: **Systemic lupus erythematosus.** *N Engl J Med* 1994, **330**:1871-1879.
3. Akira S, Uematsu S, Takeuchi O: **Pathogen recognition and innate immunity.** *Cell* 2006, **124**:783-801.
4. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, Akira S: **A Toll-like receptor recognizes bacterial DNA.** *Nature* 2000, **408**:740-745.
5. Latz E, Schoenemeyer A, Visintin A, Fitzgerald KA, Monks BG, Knetter CF, Lien E, Nilsen NJ, Espevik T, Golenbock DT: **TLR9 signals after translocating from the ER to CpG DNA in the lysosome.** *Nat Immunol* 2004, **5**:190-198.
6. Krieg AM: **CpG motifs in bacterial DNA and their immune effects.** *Annu Rev Immunol* 2002, **20**:709-760.
7. Lövgren T, Eloranta ML, Båve U, Alm GV, Rönnblom L: **Induction of interferon-alpha production in plasmacytoid dendritic cells by immune complexes containing nucleic acid released by necrotic or late apoptotic cells and lupus IgG.** *Arthritis Rheum* 2004, **50**:1861-1872.
8. Pisetsky DS, Rönnblom L: **Systemic lupus erythematosus: a matter of life and death.** *Arthritis Rheum* 2009, **60**:1567-1570.
9. Richardson B, Scheinbart L, Strahler J, Gross L, Hanash S, Johnson M: **Evidence for impaired T cell DNA methylation in systemic lupus erythematosus and rheumatoid arthritis.** *Arthritis Rheum* 1990, **33**:1665-1673.
10. Papadimitraki ED, Choulaki C, Koutala E, Bertsias G, Tsatsanis C, Gergianaki I, Raptopoulou A, Kritikos HD, Mamalaki C, Sidiropoulos P, Boumpas DT: **Expansion of Toll-like receptor 9-expressing B cells in active systemic lupus erythematosus: implications for the induction and maintenance of the autoimmune process.** *Arthritis Rheum* 2006, **54**:3601-3611.
11. Christensen SR, Kashgarian M, Alexopoulou L, Flavell RA, Akira S, Shlomchik MJ: **Toll-like receptor 9 controls anti-DNA autoantibody production in murine lupus.** *J Exp Med* 2005, **202**:321-331.
12. Nakano S, Morimoto S, Suzuki J, Nozawa K, Amano H, Tokano Y, Takasaki Y: **Role of pathogenic auto-antibody production by Toll-like receptor 9 of B cells in active systemic lupus erythematosus.** *Rheumatology (Oxford)* 2008, **47**:145-149.
13. Tao K, Fujii M, Tsukumo S, Maekawa Y, Kishihara K, Kimoto Y, Horiuchi T, Hisaeda H, Akira S, Kagami S, Yasutomo K: **Genetic variations of Toll-like receptor 9 predispose to systemic lupus erythematosus in Japanese population.** *Ann Rheum Dis* 2007, **66**:905-909.
14. He Z, Bateman A: **Progranulin (granulin-epithelin precursor, PC-cell-derived growth factor, acrogranin) mediates tissue repair and tumorigenesis.** *J Mol Med (Berl)* 2003, **81**:600-612.
15. Bateman A, Bennett HP: **The granulin gene family: from cancer to dementia.** *Bioessays* 2009, **31**:1245-1254.
16. Daniel R, He Z, Carmichael KP, Halper J, Bateman A: **Cellular localization of gene expression for progranulin.** *J Histochem Cytochem* 2000, **48**:999-1009.
17. He Z, Ong CH, Halper J, Bateman A: **Progranulin is a mediator of the wound response.** *Nat Med* 2003, **9**:225-229.
18. Van Damme P, Van Hoecke A, Lambrechts D, Vanacker P, Bogaert E, van Swieten J, Carmeliet P, Van Den Bosch L, Robberecht W: **Progranulin functions as a neurotrophic factor to regulate neurite outgrowth and enhance neuronal survival.** *J Cell Biol* 2008, **181**:37-41.
19. Kessenbrock K, Frohlich L, Sixt M, Lammermann T, Pfister H, Bateman A, Belaaouaj A, Ring J, Ollert M, Fassler R, Jenne DE: **Proteinase 3 and neutrophil elastase enhance inflammation in mice by inactivating antiinflammatory progranulin.** *J Clin Invest* 2008, **118**:2438-2447.
20. Park B, Buti L, Lee S, Matsuwaki T, Spooner E, Brinkmann MM, Nishihara M, Ploegh HL: **Granulin is a soluble cofactor for toll-like receptor 9 signaling.** *Immunity* 2011, **34**:505-513.
21. Bombardier C, Gladman DD, Urowitz MB, Caron D, Chang CH: **Derivation of the SLEDAI: a disease activity index for lupus patients: The Committee on Prognosis Studies in SLE.** *Arthritis Rheum* 1992, **35**:630-640.
22. Kim WU, Sreih A, Bucala R: **Toll-like receptors in systemic lupus erythematosus; prospects for therapeutic intervention.** *Autoimmun Rev* 2008, **7**:204-208.
23. Patole PS, Gröne HJ, Segerer S, Ciubar R, Balemezova E, Henger A, Kretzler M, Schlöndorff D, Anders HJ: **Viral double-stranded RNA aggravates lupus nephritis through Toll-like receptor 3 on glomerular mesangial cells and antigen-presenting cells.** *J Am Soc Nephrol* 2005, **16**:1326-1338.
24. Loser K, Vogl T, Voskort M, Lueken A, Kupas V, Nacken W, Klenner L, Kuhn A, Foell D, Sorokin L, Luger TA, Roth J, Beissert S: **The Toll-like receptor 4 ligands Mrp8 and Mrp14 are crucial in the development of autoreactive $CD8^+$ T cells.** *Nat Med* 2010, **16**:713-717.
25. Miyata M, Ito O, Kobayashi H, Sasajima T, Ohira H, Suzuki S, Kasukawa R: **CpG-DNA derived from sera in systemic lupus erythematosus enhances ICAM-1 expression on endothelial cells.** *Ann Rheum Dis* 2001, **60**:685-689.
26. Rönnelid J, Tejde A, Mathsson L, Nilsson-Ekdahl K, Nilsson B: **Immune complexes from SLE sera induce IL10 production from normal**

peripheral blood mononuclear cells by an FcγRII dependent mechanism: implications for a possible vicious cycle maintaining B cell hyperactivity in SLE. *Ann Rheum Dis* 2003, **62**:37-42.
27. Vercellino M, Grifoni S, Romagnolo A, Masera S, Mattioda A, Trebini C, Chiavazza C, Caligiana L, Capello E, Mancardi GL, Giobbe D, Mutani R, Giordana MT, Cavalla P: **Progranulin expression in brain tissue and cerebrospinal fluid levels in multiple sclerosis.** *Mult Scler* 2011, **17**:1194-1201.
28. Jüsten HP, Grünewald E, Totzke G, Gouni-Berthold I, Sachinidis A, Wessinghage D, Vetter H, Schulze-Osthoff K, Ko Y: **Differential gene expression in synovium of rheumatoid arthritis and osteoarthritis.** *Mol Cell Biol Res Commun* 2000, **3**:165-172.
29. Kessenbrock K, Dau T, Jenne DE: **Tailor-made inflammation: how neutrophil serine proteases modulate the inflammatory response.** *J Mol Med (Berl)* 2011, **89**:23-28.
30. Yin F, Banerjee R, Thomas B, Zhou P, Qian L, Jia T, Ma X, Ma Y, Iadecola C, Beal MF, Nathan C, Ding A: **Exaggerated inflammation, impaired host defense, and neuropathology in progranulin-deficient mice.** *J Exp Med* 2010, **207**:117-128.
31. Tang W, Lu Y, Tian QY, Zhang Y, Guo FJ, Liu GY, Syed NM, Lai Y, Lin EA, Kong L, Su J, Yin F, Ding AH, Zanin-Zhorov A, Dustin ML, Tao J, Craft J, Yin Z, Feng JQ, Abramson SB, Yu XP, Liu CJ: **The growth factor progranulin binds to TNF receptors and is therapeutic against inflammatory arthritis in mice.** *Science* 2011, **332**:478-484.
32. Wong CK, Wong PT, Tam LS, Li EK, Chen DP, Lam CW: **Activation profile of Toll-like receptors of peripheral blood lymphocytes in patients with systemic lupus erythematosus.** *Clin Exp Immunol* 2010, **159**:11-22.
33. Zeuner RA, Klinman DM, Illei G, Yarboro C, Ishii KJ, Gursel M, Verthelyi D: **Response of peripheral blood mononuclear cells from lupus patients to stimulation by CpG oligodeoxynucleotides.** *Rheumatology (Oxford)* 2003, **42**:563-569.
34. Yasuda K, Richez C, Maciaszek JW, Agrawal N, Akira S, Marshak-Rothstein A, Rifkin IR: **Murine dendritic cell type I IFN production induced by human IgG-RNA immune complexes is IFN regulatory factor (IRF)5 and IRF7 dependent and is required for IL-6 production.** *J Immunol* 2007, **178**:6876-6885.
35. Chun HY, Chung JW, Kim HA, Yun JM, Jeon JY, Ye YM, Kim SH, Park HS, Suh CH: **Cytokine IL-6 and IL-10 as biomarkers in systemic lupus erythematosus.** *J Clin Immunol* 2007, **27**:461-466.
36. Spronk PE, ter Borg EJ, Limburg PC, Kallenberg CG: **Plasma concentration of IL-6 in systemic lupus erythematosus; an indicator of disease activity?** *Clin Exp Immunol* 1992, **90**:106-110.
37. Eilertsen GØ, Nikolaisen C, Becker-Merok A, Nossent JC: **Interleukin-6 promotes arthritis and joint deformation in patients with systemic lupus erythematosus.** *Lupus* 2011, **20**:607-613.
38. Linker-Israeli M, Deans RJ, Wallace DJ, Prehn J, Ozeri-Chen T, Klinenberg JR: **Elevated levels of endogenous IL-6 in systemic lupus erythematosus: a putative role in pathogenesis.** *J Immunol* 1991, **147**:117-123.
39. Klashman DJ, Martin RA, Martinez-Maza O, Stevens RH: **In vitro regulation of B cell differentiation by interleukin-6 and soluble CD23 in systemic lupus erythematosus B cell subpopulations and antigen-induced normal B cells.** *Arthritis Rheum* 1991, **34**:276-286.
40. Nagafuchi H, Suzuki N, Mizushima Y, Sakane T: **Constitutive expression of IL-6 receptors and their role in the excessive B cell function in patients with systemic lupus erythematosus.** *J Immunol* 1993, **151**:6525-6534.
41. Kitani A, Hara M, Hirose T, Norioka K, Harigai M, Hirose W, Suzuki K, Kawakami M, Kawagoe M, Nakamura H: **Heterogeneity of B cell responsiveness to interleukin 4, interleukin 6 and low molecular weight B cell growth factor in discrete stages of B cell activation in patients with systemic lupus erythematosus.** *Clin Exp Immunol* 1989, **77**:31-36.
42. Weckerle CE, Franek BS, Kelly JA, Kumabe M, Mikolaitis RA, Green SL, Utset TO, Jolly M, James JA, Harley JB, Niewold TB: **Network analysis of associations between serum interferon-α activity, autoantibodies, and clinical features in systemic lupus erythematosus.** *Arthritis Rheum* 2011, **63**:1044-1053.

doi:10.1186/ar4087
Cite this article as: Tanaka *et al.*: Serum progranulin levels are elevated in patients with systemic lupus erythematosus, reflecting disease activity. *Arthritis Research & Therapy* 2012 14:R244.

blood

2012 119: 2263-2273
Prepublished online January 18, 2012;
doi:10.1182/blood-2011-04-351965

CIN85 is required for Cbl-mediated regulation of antigen receptor signaling in human B cells

Hiroaki Niiro, Siamak Jabbarzadeh-Tabrizi, Yoshikane Kikushige, Takahiro Shima, Kumiko Noda, Shun-ichiro Ota, Hirofumi Tsuzuki, Yasushi Inoue, Yojiro Arinobu, Hiromi Iwasaki, Shinji Shimoda, Eishi Baba, Hiroshi Tsukamoto, Takahiko Horiuchi, Tadayoshi Taniyama and Koichi Akashi

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.

CIN85 is required for Cbl-mediated regulation of antigen receptor signaling in human B cells

Hiroaki Niiro,[1] Siamak Jabbarzadeh-Tabrizi,[1] Yoshikane Kikushige,[2] Takahiro Shima,[1] Kumiko Noda,[1] Shun-ichiro Ota,[1] Hirofumi Tsuzuki,[1] Yasushi Inoue,[1] Yojiro Arinobu,[2] Hiromi Iwasaki,[2] Shinji Shimoda,[1] Eishi Baba,[1] Hiroshi Tsukamoto,[1] Takahiko Horiuchi,[1] Tadayoshi Taniyama,[3] and Koichi Akashi[1]

[1]Department of Medicine and Biosystemic Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; [2]Center for Cellular and Molecular Medicine, Kyushu University Hospital, Fukuoka, Japan; and [3]Laboratory of Bacterial Infection and Immunity, Department of Immunology, National Institute of Infectious Diseases, Tokyo, Japan

The aberrant regulation of B-cell receptor (BCR) signaling allows unwanted B cells to persist, thereby potentially leading to autoimmunity and B-cell malignancies. Casitas B-lineage lymphoma (Cbl) proteins suppress BCR signaling; however, the molecular mechanisms that control Cbl function in human B cells remain unclear. Here, we demonstrate that CIN85 (c-Cbl interacting protein of 85 kDa) is constitutively associated with c-Cbl, Cbl-b, and B-cell linker in B cells. Experiments using CIN85-overexpressing and CIN85-knockdown B-cell lines revealed that CIN85 increased c-Cbl phosphorylation and inhibited BCR-induced calcium flux and phosphorylation of Syk and PLCγ2, whereas it did not affect BCR internalization. The Syk phosphorylation in CIN85-overexpressing and CIN85-knockdown cells was inversely correlated with the ubiquitination and degradation of Syk. Moreover, CIN85 knockdown in primary B cells enhanced BCR-induced survival and growth, and increased the expression of BcLxL, A1, cyclin D2, and myc. Following the stimulation of BCR and Toll-like receptor 9, B-cell differentiation-associated molecules were up-regulated in CIN85-knockdown cells. Together, these results suggest that CIN85 is required for Cbl-mediated regulation of BCR signaling and for downstream events such as survival, growth, and differentiation of human B cells. (*Blood*. 2012;119(10):2263-2273)

Introduction

B-cell receptor (BCR) signaling guides critical cell fate decisions in B cells during ontogeny.[1,2] BCRs can generate tolerogenic signals to purge or silence B cells that bind to self-antigens, and immunogenic signals to expand B cells that are specific for foreign antigens. Thus, BCR signaling must be properly regulated at the various stages of B-cell development, as aberrant regulation of BCR signaling potentially leads to autoimmunity and B-cell malignancies.

On BCR ligation by antigens, the Src-family protein tyrosine kinase (PTK) Lyn and Syk are initially activated. Syk propagates the signal by phosphorylating downstream signaling molecules, causing the activation of critical signaling intermediates phosphoinositol 3-kinase (PI3K) and phospholipase C (PLC)γ2. PI3K activates Akt kinase, which is important for B-cell survival.[3] PLCγ2 activation induces the release of intracellular Ca^{2+} and the activation of protein kinase C (PKC), which cause the activation of mitogen-activated protein kinases (MAPKs; ERK, JNK, and p38 MAPK) and of transcription factors, including NF-κB and NF-AT. These molecules regulate further downstream molecules that are responsible for determining B-cell fates such as survival, growth, and differentiation.[1,2]

Casitas B-lineage lymphoma (Cbl) proteins are E3 ubiquitin ligases that regulate signals of various receptors by promoting the ubiquitination of signaling components.[4,5] Tyrosine phosphorylation of Cbl proteins is critical for their function.[6] Mammalian Cbl proteins consist of 3 members, c-Cbl, Cbl-b, and Cbl-3, among which c-Cbl and Cbl-b are expressed in hematopoietic cells.[7] In B cells, Cbl proteins associate with Syk and B-cell linker (BLNK), and negatively regulate BCR signaling.[8,9] B cell-specific ablation of c-Cbl/Cbl-b proteins in mice causes aberrant BCR signaling as well as impaired B-cell anergy, culminating in the development of systemic lupus erythematosus (SLE)–like disease.[10] In addition, c-Cbl is hypophosphorylated on tyrosine in advanced stages of chronic lymphocytic leukemia (CLL).[11] These findings suggest that Cbl-mediated regulation of BCR signaling is critical for the fate decisions of self-reactive and malignant B cells.

Adaptors are noncatalytic molecules that integrate the spatial and temporal assembly of multiprotein complexes involved in the survival, growth, and differentiation of B cells. We previously showed that the B lymphocyte adaptor molecule of 32 kDa (Bam32)/DAPP1 regulates BCR signaling/internalization and B-cell survival.[12,13] The *SH3KBP1* (SH3-domain kinase-binding protein 1) gene, which is also known as CIN85 (c-Cbl interacting protein of 85 kDa), encodes an adaptor that is independently identified by several groups and contains 3 SH3 domains, a proline-rich region, and a coiled-coil domain.[14-17] Early studies showed that in nonimmune cells, CIN85 regulates the clathrin-dependent internalization of receptor tyrosine kinases (RTKs) such as epidermal growth factor receptors (EGFRs).[18,19] The formation of the ternary complex of CIN85, c-Cbl, and endophillin is critical for this process. In immune cells, however, little is known approximately the function of CIN85. CIN85 facilitates ligand-induced FcεRI internalization in RBL-2H3 mast cells.[20] In addition, it regulates FcεRI signaling via Cbl-mediated regulation of

Submitted April 29, 2011; accepted January 13, 2012. Prepublished online as *Blood* First Edition paper, January 18, 2012; DOI 10.1182/blood-2011-04-351965.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

Syk expression in RBL-2H3 cells.[21] A recent study showed that CIN85 modulates c-Cbl–mediated down-regulation of FcγRIIa in human neutrophils.[22] It is thus of interest to determine whether CIN85 regulates the signaling pathways of other multimeric immune receptors, such as the T- and B-cell receptors.

Here, we demonstrate that CIN85 is constitutively associated with c-Cbl, Cbl-b, and BLNK in human B cells. Gain-of-function and loss-of-function experiments revealed that CIN85 up-regulated c-Cbl phosphorylation and inhibited BCR-induced calcium flux and phosphorylation of Syk and PLCγ2, without affecting BCR internalization. CIN85 also promoted c-Cbl–dependent ubiquitination and degradation of Syk. Moreover, CIN85 knockdown in primary B cells caused enhanced BCR-induced survival and growth, and augmented BCR/TLR9-induced expression of B-cell differentiation-associated molecules. Collectively, these results suggest that CIN85 is required for Cbl-mediated regulation of BCR signaling and for downstream events such as the survival, growth, and differentiation of human B cells.

Methods

Reagents

Goat anti–human IgM and IgG/IgA/IgM F(ab′)$_2$ fragments were purchased from Jackson ImmunoResearch Laboratories. Rabbit anti–human phospho-Zap-70 (Y319)/Syk (Y352), anti–human phospho-PLCγ2 (Y1217), anti–human phospho-Akt, anti–mouse Akt, anti–human phospho-JNK, anti–human phospho-ERK, and anti–human PLCγ2 pAbs as well as anti–human BclxL and Blimp-1 mAbs were purchased from Cell Signaling Technology. Mouse anti–human phospho-Btk, anti–human phospho-BLNK, anti–human c-Cbl, and anti–human Rac1 mAbs were from BD Immunocytometry. Mouse anti–human Cbl-b, anti–human Syk (4D10), anti–human BLNK, and anti-ubiquitin mAbs as well as rabbit anti–human c-Cbl and anti–mouse cyclin D2 pAbs were from Santa Cruz Biotechnology. Mouse anti-V5 mAb was from Invitrogen. Mouse anti-phosphotyrosine and anti–human CIN85 mAbs were from Upstate Biotechnology. Rabbit anti–human Vav2 mAb was from Epitomics. Sheep anti–human CD2AP pAb was from R&D Systems. Mouse anti–β-actin mAb was from Sigma-Aldrich.

B cell lines and primary B cells

The B lymphoma cell line BJAB was cultured in RPMI 1640 medium supplemented with 10% FCS. Human peripheral blood mononuclear cells, kindly provided by healthy volunteers, were separated from their buffy coats. Informed consent was obtained from all subjects in accordance with the Declaration of Helsinki. The Institutional Review Board of Kyushu University Hospital approved all research on human subjects. B cells were isolated with Dynabeads M450 CD19 and DETACHaBEAD CD19 (Dynal Biotech), according to the manufacturer's instructions. The isolated B cells exhibited greater than 99.5% viability on trypan blue exclusion and more than 95% purity on flow cytometry. Trace levels of phosphorylation of BCR-signaling molecules were observed in the B cells immediately after purification, probably because of mechanical stress.[23] The cells were thus rested for a couple of hours before further analysis. The cells were cultured at a density of 1×10^6 cells/mL in a 96 flat-bottom microtiter plate in complete RPMI 1640 medium supplemented with 10% FCS.

Expression constructs and transfection

Constructs encoding V5-tagged wild-type (WT) and 3 SH3 domain-deleted mutants of human CIN85 (pEF1/V5-CIN85 and –CIN85-dSH3ABC) were previously described.[24] The BJAB cells were transfected with the aforementioned constructs using a Gene Pulser apparatus (Bio-Rad Laboratories). The control cells were transfected with an empty vector. Stably transfected BJAB clones were selected in the presence of G418 (2 mg/mL) and screened with anti-V5 mAb.

RNA interference

The pSUPER-based strategy was adopted to knockdown hCIN85 expression. To generate CIN85 small-hairpin RNA (shRNA), a 19-nucleotide sequence (CAGCAATGACATTGACTTA) selected from human CIN85 cDNA was annealed and ligated into the pSUPER or GFP-pSUPER vector. A scrambled sequence (GTTACTAACGCGAATTAAC) was used as negative control. hCIN85 or the control shRNA vector was transfected into BJAB cells using a Gene Pulser apparatus, and stable hCIN85-knockdown BJAB clones were selected in the presence of puromycin (0.5 μg/mL). Transient transfections of primary B cells with the pSUPER-hCIN85 vector were performed using the Nucleofection kit from AMAXA Biosystems as previously described.[23]

Measurement of intracellular free calcium

B cells were washed with RPMI 1640 medium containing 10% FCS and adjusted to a concentration of 1×10^6 cells/mL. After incubation at 37°C for 15 minutes, 1 μg/mL of Fluo 4/AM (Dojindo) was added, and the cells were incubated for an additional 30 to 45 minutes with resuspension every 15 minutes. The cells were centrifuged and resuspended in RPMI 1640 at a density of 2×10^6 cells/mL and stimulated with anti-IgM (20 μg/mL). The fluorescence intensity of intracellular Fluo 4 was monitored and analyzed using flow cytometry.

Immunoprecipitation

Cells were lysed as described.[13] Subsequently, protein G-Sepharose (Amersham Pharmacia Biotech) precleared lysates were incubated with anti-V5, -BLNK, -Syk, -Cbl, -Vav2 mAb, or -CD2AP pAb for 1 hour at 4°C and then immunoprecipitated with protein G-Sepharose overnight at 4°C. The precipitated proteins were resolved by 10% SDS-PAGE; transferred onto a Millipore Immobilon polyvinylidene difluoride membrane; and blotted with anti-phosphotyrosine (4G10), -V5, -c-Cbl, -Cbl-b, -BLNK, -Vav2, -Syk, or -ubiquitin mAbs, followed by incubation with secondary HRP-conjugated IgG (Jackson ImmunoResearch Laboratories) specific for the primary Ab. The blots were developed with an ECL Plus kit (Amersham Biosciences).

Western blot analysis

Nonstimulated or stimulated cells (1×10^6) were lysed as described.[12] The lysates were then denatured in an equal volume of 2 × SDS sample buffer, resolved on a 10% SDS-PAGE gel, and electro-transferred to nitrocellulose membranes in non-SDS–containing transfer buffer (25mM Tris, 0.2M glycine, and 20% methanol; pH 8.5). Western blotting was performed with anti–phospho-Syk (1:2000), anti–phospho-PLCγ2 (1:2000), anti–phospho-BLNK (1:2000), anti–phospho-JNK (1:2000), anti–phospho-ERK (1:2000), anti–phospho-Akt (1:2000), anti-Akt (1:2000), anti-CIN85 (1:2000), anti–β-actin (1:2000), or anti-Vav2 (1:2000), followed by a 1:15 000 dilution of anti–rabbit or anti–mouse HRP-conjugated IgG. The blots were developed with the ECL plus kit (Amersham Biosciences). The chemiluminescence intensity was monitored using a Laser3000 (FujiFilm) instrument. We quantitated the band intensity of the proteins using ImageGauge Version 4.22 software (FujiFilm). The resulting values were expressed as fold changes in protein expression relative to the protein expression in unstimulated control cells.

Luciferase assays

Cells (1×10^7) were transfected by electroporation with the NF-AT-reporter construct, which was generously provided by Dr Shoichiro Miyatake (The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). After 18 to 20 hours, cells were harvested and plated on 96-well plates at a density of 2×10^5/well. Triplicate cultures were incubated in the media alone with graded doses of anti-IgM or with 50nM PMA and 2.5μM ionomycin. After 6 hours, the cells were lysed in 50 μL reporter lysis buffer (Promega) for 15 minutes at room temperature. The luciferase activity was assayed by adding 20 μL luciferase substrate (Promega) to 50 μL lysate