Concise report

Dysregulated mature IL-1β production in familial Mediterranean fever

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Abstract

Objective. The aim of this study was to analyse the role of circulating cleaved IL-1β in patients with FMF.

Methods. We enrolled 20 patients with FMF (5 males and 15 females), 22 patients with RA (4 males and 18 females) and 22 healthy controls (6 males and 16 females). Serum levels of serum amyloid A (SAA) were measured by ELISA. We also determined whether IL-1β was present as the cleaved form (p17) in the sera of FMF patients by immunoblotting using anti-cleaved IL-1β antibody.

Results. Although SAA concentrations were elevated in the sera, there was no significant difference in these concentrations between FMF patients and RA patients. Immunoblot analysis demonstrated that the cleaved form of IL-1β (p17) was present in sera from FMF patients during febrile attack periods, but not in healthy controls. Bands representing the cleaved form of IL-1β were not detected in serum from FMF patients at non-febrile attack periods or remission periods under colchicine treatment. The amounts of cleaved IL-1β (p17) were significantly higher in patients with FMF compared with those in patients with RA in the inflammatory phase.

Conclusion. The cleaved form of IL-1β is a valuable biomarker for monitoring disease activity and response to colchicine treatment in patients with FMF. It might be useful to discriminate FMF from other non-IL-1β-mediated inflammatory disorders.

Key words: IL-1β, familial Mediterranean fever, biomarker, autoinflammatory disease, serum amyloid A.

Introduction

FMF is the most common human hereditary autoinflammatory disorder, characterized by recurrent, self-limited bouts of fever and localized inflammation, usually involving the peritoneum, pleura and joints [1]. These episodes of inflammation are caused mainly by a massive influx of neutrophils into serous cavities and are accompanied by elevation of acute phase reactants [2]. The effectiveness of anti-IL-1β treatment has suggested that IL-1β plays a role in the pathophysiology of the disease [3]. However, dysregulated IL-1β induction in patients with FMF has been demonstrated in few studies [4].

FMF is caused by mutations within the Mediterranean fever (MEFV) gene, which encodes pyrin, a protein that has been proposed to exert a suppressive effect on inflammasome activation [5]. This inhibitory control mechanism is lost through mutations within the MEFV gene [6]. This hypothesis is supported by ex vivo evidence where mononuclear cells isolated from patients with FMF showed enhanced IL-1β production [4]. However, actual serum levels of IL-1β have not been reported, indicating a disparity with the in vitro findings. Furthermore, the role of IL-1β in the pathogenesis of FMF and its measurement...
in serum during acute attacks as well as the mediators that trigger acute inflammation in FMF have not been elucidated. The production and biological activity of IL-1β are regulated at multiple levels, including transcription, translation, cleavage and release [7]. Inflammasome activation leads to the conversion of pro-caspase-1 into caspase-1 and subsequent cleavage of pro-IL-1β into mature IL-1β, which is secreted from cells [8].

As described above, measurement of serum IL-1β is not a reliable indicator of IL-1β-mediated diseases [9], therefore the direct detection of the mature form of IL-1β is critical in IL-1β-mediated inflammation. We conducted this study to measure the serum levels of the mature form of IL-1β in FMF patients with and without acute attacks compared with control subjects.

Methods

Subjects

Twenty Japanese patients with FMF were evaluated and compared with 22 healthy subjects [6 males, 16 females; mean age 34.7 years (s.d. 7.8)] and 22 patients with RA [4 males, 18 females; mean age 51.5 years (s.d. 15.1)] as controls. FMF patients were diagnosed clinically according to the Tel-Hashomer criteria [10] and all patients exhibited a favourable response to colchicine. The mean age of FMF patients (5 males and 15 females) was 34.9 years (s.d. 11.1). The distribution of the MEFV genotype was as follows: M694I/M694I, 3; M694I/-, 2; M694I/E148Q, 4; E148Q/-, 1; L110P/E148Q/E148Q, 1; L110P/E148Q, 1; L110P/E148Q/R202Q, 3; E148Q/P369S, 1; E148Q/P369S/R408Q, 1; G304R/-, 1; no mutation, 2. In four patients with FMF, serial analysis was performed using sera obtained during febrile attacks and symptom-free periods under colchicine treatment. At the time of sample collection, seven patients were receiving colchicine treatment to control clinical manifestations. The present study was approved by the ethics committee of Nagasaki Medical Center [no. 21015 (2009)]. MEFV genetic analysis was also approved by the ethics committee of Nagasaki Medical Center [no. 21003 (2009)]. Written informed consent was obtained from each individual included in this study.

Immunoblot analysis

Human serum was diluted 10-fold with PBS. We separated 1.5 μl of this diluted human serum sample plus 5 μl of protein loading buffer under reducing conditions by NuPAGE 3–8% Tris-acetate gel electrophoresis (Invitrogen, Carlsbad, CA, USA). Proteins were electrophoretically transferred onto an Invitrogen polyvinylidene fluoride membrane and incubated overnight at 4°C with blocking solution [5% non-fat milk in Tris-buffered saline with 0.05% Tween 20 (TTBS)]. The blocked membrane was incubated with rabbit anti-human cleaved IL-1β polyclonal antibody (MyBioSource, San Diego, CA, USA; 1:10 000 dilution with 1% non-fat milk in TTBS) for 1 h at room temperature and then washed five times with TTBS buffer for 10 min each time at room temperature with constant shaking. Then the membrane was incubated with horseradish peroxidase-conjugated second antibody (1:3000 dilution; Santa Cruz Biotechnology, Dallas, TX, USA) for 1 h at room temperature and washed five times with TTBS buffer for 10 min each time at room temperature with constant shaking. Immunodetection analysis was performed using an electrochemiluminescence western blotting kit (Amersham, Little Chalfont, UK).

Images of the developed film were scanned using an LAS-3000 image analyser (Fujifilm, Tokyo, Japan). The densities of the cleaved IL-1β bands with a molecular weight (MW) of 17 kDa were measured using ImageJ software (Wayne Rasband, Bethesda, MD, USA) and the related ratio to the amount of immunoglobulin light chain was calculated through the use of standards run on each gel.

Measurement of serum amyloid A

Serum amyloid A (SAA) concentrations were assayed using an ELISA kit from BioSource International (Camarillo, CA, USA).

Statistics

Differences between groups were examined for statistical significance using Mann–Whitney U test. P-values < 0.05 were considered statistically significant. Analysis was performed using SPSS version 18 software (IBM, Armonk, NY, USA).

Results

SAA concentrations in patients with FMF and RA

SAA concentrations were measured in sera from FMF patients, RA patients and healthy subjects (Fig. 1A). Serum concentrations of SAA were markedly elevated in FMF [206.5 mg/ml (s.d. 234.7)] and RA patients [237.7 mg/ml (s.d. 256.4)] compared with healthy subjects [7.5 mg/ml (s.d. 5.4)]. However, there was no significant difference in circulating SAA levels between FMF and RA patients.

Cleaved IL-1β in sera from FMF patients during febrile attack periods

Serum samples from a patient with newly diagnosed FMF (untreated) were subjected to immunoblot analysis using anti-cleaved IL-1β antibodies. Previous studies indicated that IL-1β binds with large molecular mass proteins, such as α2-macroglobulin, through a thiol-disulphide bridge in the plasma [11]. The protein–IL-1β complex had a MW of ~400 000 kDa in non-reducing SDS-PAGE but dissociated when exposed to β-mercaptoethanol [12]. To support this, free cleaved IL-1β with a MW of 17 kDa was not detected in the sera by immunoblot analysis using anti-cleaved IL-1β antibodies under non-reducing conditions. However, cleaved IL-1β was detected in serum from a patient with typical FMF under reducing conditions (Fig. 1B), indicating that cleavage of the internal thiol-ester could enable the occurrence of circulating cleaved IL-1β in the sera. We also analysed sera from the same patient during the febrile and non-febrile attack periods.

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Fig. 1 Anti-cleaved IL-1β immunoblot analysis

(A) Serum SAA levels by ELISA. SAA serum levels were measured in patients with FMF or RA and healthy subjects. Closed triangles: FMF patients with MEFV exon 10 mutations. Open triangles: FMF patients without MEFV exon 10 mutations. There was no significant difference in SAA levels between patients with FMF and those with RA. (B) Anti-cleaved IL-1β immunoblot analysis using sera from an FMF patient. Sera from an FMF patient during the febrile attack period and recombinant IL-1β (10 ng, cleaved form) were dissolved in sample buffer under non-reducing or reducing conditions and separated in Tris-acetate gels. The cleaved IL-1β band (p17) was detected in serum from an FMF patient under reducing conditions. Additional bands observed under reducing conditions are light chains generated from cleavage of the thiol-disulphide bridge of immunoglobulins. A representative result of three independent experiments. (C) Cleaved IL-1β (p17) bands in an FMF patient during the febrile attack period. Sera from the same FMF patient during the febrile attack period and non-febrile attack period and two healthy subjects were analysed by anti-cleaved IL-1β immunoblot analysis. The concentrations of SAA are shown in the lower panel of each sample. A representative result of three independent experiments. (D) Cleaved IL-1β (p17) bands in four patients with FMF during the febrile and non-febrile attack periods. Sera from four patients with FMF during the febrile and non-febrile attack periods were analysed by anti-cleaved IL-1β immunoblot analysis. A representative result of two independent experiments. SAA: serum amyloid A.
As shown in Fig. 1C, cleaved IL-1β was detected in sera during the febrile attack period but not at the non-febrile attack period, nor in sera from healthy subjects. The detection of cleaved IL-1β was parallel to the elevated levels of SAA.

Resolution of cleaved IL-1β in sera from patients controlled by colchicine

We examined the changes in circulating cleaved IL-1β during febrile attack phases and remission phases by colchicine treatment in the same FMF patient. As shown in Fig. 1D, serum cleaved IL-1β bands in the febrile attack phase were eliminated in the non-febrile attack phase in patients with FMF.

Circulating cleaved IL-1β in patients with FMF and RA

Analyses were performed in patients with FMF and RA. As shown in Fig. 2A, cleaved IL-1β was detected exclusively in FMF patients with elevated SAA levels, indicating that FMF-mediated inflammation resulted in the occurrence of circulating IL-1β. In contrast to FMF patients, cleaved IL-1β was barely detected in RA patients with elevated serum SAA levels (Fig. 2B). To quantify the circulating cleaved form of IL-1β, we calculated the ratio of cleaved IL-1β bands against standard immunoglobulin light chain bands in FMF patients (n = 14) and RA patients (n = 17) with elevated SAA levels (>8 μg/ml), who were presumed to be in the pro-inflammatory phase. As shown in Fig. 2C, the mean ratio of cleaved IL-1β bands to standard immunoglobulin light chains was significantly elevated in FMF patients [0.064 (S.D. 0.033)] compared with RA patients [0.010 (S.D. 0.007)], despite the same levels of SAA. However, there was no significant difference in the ratio of cleaved IL-1β between FMF patients with or without MEFV exon 10 mutation [patients with exon 10 mutation, 0.067 (S.D. 0.034) vs patients without exon 10 mutation, 0.061 (S.D. 0.033)].

Discussion

FMF is a hereditary autoinflammatory disease characterized by spontaneous activation of the innate immune system [1]. Fever and acute abdominal pain are the most common symptoms, but disease manifestations also include arthritis, pleuritis, localized erythema and amyloidosis [1, 2]. The inflammation observed in FMF patients is characterized by neutrophil influx to peripheral tissues and increased serum levels of acute phase reactants [2, 4]. Genetic analysis can verify the clinical diagnosis of FMF, however, the impact of many mutations on disease expression is still a matter of debate [1]. The ideal tool to monitor inflammation should be directed towards the pathophysiological processes of the disease. Pharmacological inhibitors of IL-1β/IL-1 receptor signalling are efficacious in the treatment of FMF, supporting the hypothesis that the pathophysiology of FMF is mediated in part by IL-1β [13]. High levels of IL-8, IL-6 and TNF-α were found in sera of patients with FMF who had not been treated with colchicine [14]. Surprisingly, elevated levels of serum IL-1β, the cytokine thought to contribute most to the pathogenesis of FMF, were not demonstrated in patients with FMF. This contrasting finding might be explained by the short half-life of this cytokine and its tight binding to membrane and soluble receptors, suggesting that IL-1β might be the initial trigger of inflammation, whereupon serum IL-1β levels return to normal [15].

The primary goal of this study was to determine specific inflammatory biomarkers in patients with FMF. We found that the cleaved form of IL-1β, p17, was detectable in the serum of FMF patients. Furthermore, this study confirmed that p17 was exclusively found in the attack phase of FMF and that it was undetectable in patients with FMF in the remission phase receiving colchicine treatment. It is striking that circulating cleaved IL-1β in patients with active FMF was clearly different from levels in patients with RA, in which there was no significant difference in circulating SAA. Effective colchicine therapy abrogated the circulating cleaved form of IL-1β in FMF patients. Taken together, the circulating cleaved form of IL-1β reflects the disease activity of the pro-IL-1β processing pathway that is mainly responsible for pathophysiological processes in FMF. This clinical observation underlines the suitability of circulating cleaved IL-1β as a marker for autoinflammation seen in FMF.

Although the significance of IL-1β in the pathogenesis of FMF is supported by the therapeutic effectiveness of IL-1 blockers [3], how mutant pyrin modulates IL-1β production is still being debated. Despite reported discrepancies in the functions of pyrin, caspase-1 activation is the hallmark of FMF [16]. It has been shown that caspase-1 activation is induced by pyrin mutations and leads to dysregulated IL-1β production [17, 18]. Unlike most cytokines, IL-1β production occurs in two steps: first, the expression of a 33 kDa IL-1 precursor, pro-IL-1β, which accumulates in the cytosol, and second, cleavage of pro-IL-1β by a specific proteolytic enzyme, caspase-1, to form a 17 kDa active form of IL-1β [7]. Previous studies investigating the functional role of pyrin, the mutated protein in FMF, have suggested that it might affect NLRP3 inflammasome activation [17]. Pyrin can interact with crucial elements of the NLRP3 inflammasome, such as apoptosis-associated speck-like protein containing a CARD (ASC) and caspase-1. Furthermore, it has been postulated that pyrin mutations could induce a loss of function in its potential inhibitory action on the NLRP3 inflammasome, resulting in pro-IL-1β activation [19].

The ideal tool to monitor inflammation should measure a molecule involved in the pathophysiological processes of FMF rather than being a surrogate marker. IL-1β may be difficult to detect in serum, as significant amounts of pro-IL-1β remain inside cells. In addition, serum IL-1β binds to target proteins such as β2-microglobulin and the soluble type II IL-1 receptor, making its detection difficult. This study represents the first attempt to characterize circulating cleaved IL-1β using sera from FMF patients. The fact that IL-1β could not be detected in sera may reflect limitations in the available assay.
however, our new method of detecting the circulating cleaved form of IL-1β adds a valuable tool for monitoring the clinical course of IL-1β-mediated disorders. Our findings underscore the clinical value of circulating cleaved IL-1β as a diagnostic tool for FMF and as a biomarker of disease activity during the course of disease.

In summary, this study illustrates the accuracy of serum p17, the cleaved form of IL-1β, as a biomarker of febrile acute inflammation in patients with FMF. Further studies are warranted to establish its role as an indicator of autoinflammation or disease activity in patients with FMF. This study is the first to measure serum cleaved IL-1β in patients with FMF and RA.

Fig. 2 Cleaved IL-1β (p17) in patients with FMF and RA

(A) Cleaved IL-1β (p17) bands in patients with FMF. Sera from seven patients with FMF were analysed by anti-cleaved IL-1β immunoblot analysis. The concentrations of SAA are shown in the lower panel of each sample. A representative result of three independent experiments. (B) Cleaved IL-1β (p17) bands in patients with RA. Sera from seven patients with RA and one patient with FMF (positive control) were analysed by anti-cleaved IL-1β immunoblot analysis. The concentrations of SAA are shown in the upper panel of each sample. A representative result of three independent experiments. (C) Distribution of the ratio of cleaved IL-1β (p17) to standard immunoglobulin light chain. The ratio of cleaved IL-1β (p17) to standard immunoglobulin light chain in sera from FMF and RA patients with elevated levels of SAA (>8 mg/ml) was calculated. Closed triangles: FMF patients with MEFV exon 10 mutations. Open triangles: FMF patients without MEFV exon 10 mutations. Mann–Whitney U test revealed that the ratio was significantly higher in FMF patients compared with RA patients. SAA: serum amyloid A.
FMF patients, although we used a relatively small sample size. Larger studies including IL-1β-mediated autoinflammatory diseases are required to further our understanding of the role of this biomarker in FMF.

**Rheumatology key messages**

- Circulating cleaved IL-1β (p17) was shown to be induced in FMF patients.
- Cleaved IL-1β (p17) is a valuable biomarker for monitoring disease activity, inflammation and response to treatment in patients with FMF.

**Funding:** This work was supported by Research on Measures for Intractable Diseases Project, a matching fund subsidy from the Ministry of Health, Labor and Welfare, Japan.

**Disclosure statement:** The authors have declared no conflicts of interest.

**References**