Plasma levels of IL-1β and IL-37 in patients with severe haemophilia

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Abstract

Objective: Haemophilia A and B are disorders caused by the lack of clotting factors VIII and IX, respectively. Repeated bleeding into the same joint leads to haemophilic arthropathy (HA). Interleukin (IL)-1β is responsible for the pro-inflammatory response and IL-37 is induced by IL-1β stimuli to have an anti-inflammatory response and prevent uncontrolled inflammation and tissue damage. Our objective was to investigate plasma levels of IL-1β and IL-37 in patients with severe haemophilia with different severities of HA.

Methods: Peripheral blood samples were collected from 14 patients with severe haemophilia A and 6 with severe haemophilia B, and 18 healthy individuals. Plasma levels of IL-1β and IL-37 were detected by immunoassay, and severity of HA was evaluated using the Pettersson scoring system. Plasma levels of IL-1β and IL-37 were analysed in patients with severe haemophilia grouped by Pettersson score and in healthy individuals.

Results: Plasma levels of IL-1β and IL-37 were significantly higher in patients with severe haemophilia compared with healthy individuals and significantly lower in those with moderate to severe HA than in those with no or mild HA.

Conclusions: Plasma levels of IL-1β and IL-37 may be useful to track HA progression in patients with severe haemophilia.

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Introduction

Haemophilia is an X-linked bleeding disorder in which the blood clotting process is slowed.[1,2] Haemophilia A and B are caused by deficiency of clotting factors VIII and IX, respectively.[1,3,4] Based on the plasma levels of clotting factors, haemophilia can be classified as severe (factor levels <1%), moderate (factor levels 1% to 5%), or mild (factor levels >5% to 30%).[5] Haemophilic arthropathy (HA) is the most typical manifestation caused by repeated bleeds in patients with haemophilia.[6–8] Repeated bleeding into the same joint causes pain, progressive rigidity, marked limitation of motion, muscle wasting, and osteoporosis, leading to chronic proliferative synovitis, cartilage degeneration, bone degeneration, and eventually impaired joint function.[7] The treatment of acute haemarthrosis is limited to early replacement of clotting factor concentrate combined with rest until the pain subsides. No specific therapies have been developed for preventing damage caused by blood already present in the joints.[9]

In 2015, Van Vulpen et al.[10] demonstrated that interleukin (IL)-1β is the main mediator of cartilage damage. Blocking IL-1β can prevent chondrocyte apoptosis and cartilage damage. These findings indicate new possibilities for the prevention of blood-induced joint damage in haemophilia. In addition, several IL-1β blockers, including anakinra (a recombinant IL-1 receptor antagonist), rilonacept (an anti-IL-1β dimeric glycoprotein), and canakinumab (a recombinant human anti-IL-1β monoclonal antibody), have been clinically used to treat arthritis caused by gout.[11] IL-1β belongs to the IL-1 family (IL-1F), which has 11 members and 10 receptors.[7,12] However, the expression levels of members of this family in haemophilia have been rarely investigated.

IL-37 is an IL-1F cytokine discovered by Kumar et al. in 2000.[13,14] The main function of IL-37 is anti-inflammatory[12] and IL-37 has been found to play an important role in a variety of inflammatory diseases, autoimmune diseases, and tumours.[14] IL-37 is not constitutively expressed in blood monocytes of healthy individuals but is induced by pro-inflammatory stimuli, such as IL-1β;[15] the activation of IL-37 is a protective mechanism to prevent uncontrolled inflammation and excessive tissue damage.

Rheumatoid arthritis (RA) is a chronic, systemic, autoimmune disease characterized mainly by joint inflammation and destruction.[14] A variety of cytokines are involved in the pathogenesis of RA, including IL-37. Serum levels of IL-37 are significantly higher in patients with RA than in healthy individuals and are closely related to disease activity (C-reactive protein, erythrocyte sedimentation rate, and Disease Activity Score-28).[14,16] After treatment with disease-modifying antirheumatic drugs, IL-37 levels are decreased in drug-susceptible patients.[14,16–18] In vitro experiments have shown that IL-37 significantly suppresses the expression of IL-1β.[19] An elevated IL-37 level has also been observed in patients with erosive inflammatory osteoarthritis (OA) and is positively correlated with disease activity and pro-inflammatory cytokines such as IL-1β, tumour necrosis factor (TNF)-α,

and IL-6 in peripheral blood mononuclear cells and synovial cells. In addition, recombinant IL-37 can suppress expression of pro-inflammatory cytokines.[20] Intra-articular injections of recombinant IL-37 or adenovirus encoding human IL-37 in mice with collagen-induced or streptococcal cell wall-induced arthritis have been shown to ameliorate arthritic symptoms.[20–22]

HA has the inflammatory characteristics of RA and the degenerative features of OA.[1,3] In RA and OA, many cellular constituents and plasma constituents, including cytokines, have been detected and considered potentially responsible for HA.[1] However, the expression level of IL-37 in HA has been not investigated. In this study, we grouped patients with severe haemophilia according to severity of HA. Then, we detected and compared plasma levels of IL-1β and IL-37 between healthy individuals, patients with severe haemophilia, and groups of patients with different severities of HA.

Materials and Methods

Ethical approval

This study was approved by the Institutional Review Board of Kaohsiung Medical University Hospital (KMUHIRB-SV(II)-20150094). Written informed consent was obtained from each participant.

Patient enrolment

Peripheral blood samples were collected from patients with severe haemophilia A (n = 14), f 10 to 54 (29.27 ± 3.43) years or with severe haemophilia B (n = 6), aged 7 to 37 (23.13 ± 4.18) years. Of these, two patients received primary prophylaxis that started before the age of 3 years; two patients had on-demand treatment followed by secondary prophylaxis since the age of

14 and 26 years; and the others had on-demand treatment. The severity of HA was evaluated using the Pettersson scoring system. Ankles, knees, and elbows are hinge joints, which are the major joints affected in HA.[23,24] Therefore, we used Pettersson scores of these six joints (bilateral elbows, bilateral ankles, and bilateral knees) to classify the severity of HA in our patients. The Pettersson scoring system used the following items to evaluate HA: orthopaedic changes in osteoporosis, enlarged epiphysis, irregular subchondral surface, narrowing of joint space, subchondral cyst formation, erosion of joint margins, gross incongruence of articulating bone ends, and joint deformity. Each joint was scored between 0 and 13 points, where score 0 represents no arthropathy, score 1 to 4 represents mild arthropathy, and score 5 to 13 represents moderate to severe arthropathy.[25] Therefore, we use a score of 5 to divide patients into two groups. Patients with a Pettersson score <5 for each joint were grouped into group A, whereas patients with a Pettersson score ≥5 for any joint were grouped into group B. Our participants did not have a history of hepatitis B virus infection or hypertension. Although 10 of 20 patients had been infected with hepatitis C virus (HCV), they were treated and negative for HCV RNA in their blood samples when they were enrolled in this study. In addition, healthy volunteers (n = 18) with no history of arthritis, aged 23 to 54 (33.88 ± 1.95) years, were randomly enrolled as controls.

Immunoassays

Venous peripheral blood of patients with haemophilia and healthy individuals was collected using ethylenediaminetetraacetic acid as the anticoagulant agent. Plasma was obtained through centrifugation at $1000 \times g$ at 4°C for 10 minutes and stored at −80°C until use. Plasma levels of IL-1β

and IL-37 were determined using the ProCartaPlex immunoassay (PPX-05-MXYGRG6; Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. The sensitivity limit was 1.99 pg/mL for IL-1β and 4.22 pg/mL for IL-37. Frozen samples were thawed on ice and mixed well by vortexing, followed by centrifugation at $10,000 \times g$ for 10 minutes. Then, 25 μL of 1$\times$ Universal Assay Buffer with magnetic beads was added to dedicated wells, followed by 25 μL of standard or sample, and the plates were incubated with shaking at room temperature for 60 to 120 minutes. For the wash steps, 150 μL of wash buffer was added to each well and incubated for 30 s. The wash buffer was removed from the wells by quickly inverting the plate and blotting it on absorbent paper to remove any residual solution. Then, 25 μL of detection antibody solution was added to each well and incubated with shaking at room temperature for 30 minutes. A wash step was performed to remove excess antibody and 50 μL of streptavidin-phycoerythrin (PE) was added to each well and incubated with shaking at room temperature for 30 minutes. A wash step was performed to remove excess streptavidin-PE. Finally, 120 μL of reading buffer was added to the mixture and shaken at room temperature for 5 minutes before the data were acquired on a Luminex 200 system multiplex analyser (Luminex Corp., Austin, TX, USA).

Statistical analysis

Statistical analyses were performed using GraphPad Prism Version 5 (GraphPad Prism Software, Los Angeles, CA, USA). Measurement data are expressed as mean $\pm$ standard error of the mean (SEM). The unpaired Student's t test was used to determine intergroup differences, and linear regression was used to determine the relationship between two continuous variables. $p < 0.05$ was considered statistically significant.

Results

IL-1β and IL-37 plasma levels in patients with severe haemophilia and healthy individuals

Plasma levels of IL-1β and IL-37 were compared between patients with severe haemophilia and healthy individuals. The IL-1β and IL-37 levels were significantly higher ($p < 0.0001$) in patients with severe haemophilia than in the healthy individuals (Figure 1).
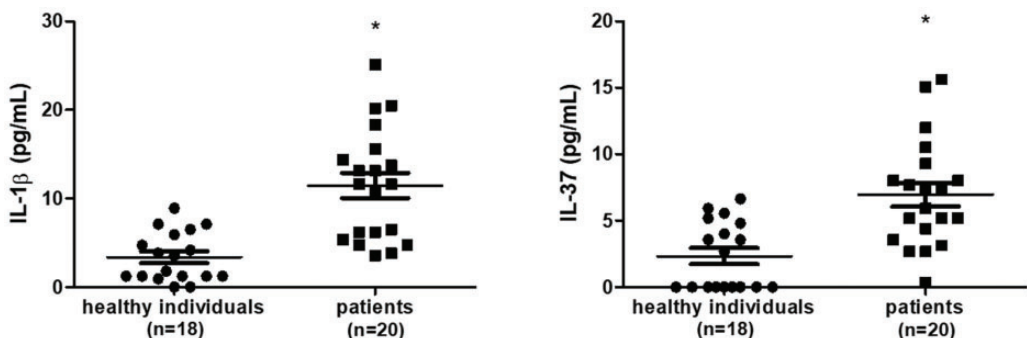

Figure 1. Analysis of plasma levels of interleukin (IL)-1β and IL-37 between patients with severe haemophilia and healthy individuals. IL-1β and IL-37 plasma levels were significantly higher in patients with severe haemophilia (n = 20) than in healthy individuals (n = 18). *$p < 0.05$.

IL-1β and IL-37 plasma levels in patients grouped by Pettersson scores

The age and Pettersson scores of six index joints per patient are listed in Table 1. Patients were grouped into Pettersson group A (no arthropathy or mild arthropathy) and Pettersson group B (moderate to severe arthropathy) as described above. In total, 120 joints of 20 patients were evaluated. The number of affected joints per patient ranged from 0 to 6. In group A, five of eight patients had no affected joint, two patients had two affected joints, and one patient had one affected joint. In group B, two patients had six affected joints, two patients had five affected joints, two patients had four affected joints, five patients had three affected joints, and one patient had one affected joint. The average numbers of affected joints per patient of group A and B were 0.625 and 3.83, respectively. Our results showed that the IL-1β plasma levels in group A ($p = 0.0004$) or group B ($p = 0.0007$) patients were significantly higher than those in healthy individuals. The plasma levels of IL-37 in group A ($p < 0.0001$) or group B ($p = 0.0108$) patients were significantly higher than those in healthy individuals. In addition, plasma levels of IL-1β ($p = 0.0038$) and IL-37 ($p = 0.0105$) were significantly lower in group B patients than in group A patients (Figure 2).

Correlation between cytokine levels and Pettersson scores

We analysed correlations between cytokine levels and the total Pettersson scores of six

Table 1. Patients grouped according to Pettersson score (indicating severity of haemophilic arthropathy) in six joints.

Haemophilia type	Age (years)	Pettersson score						Sum	Group
		Right elbow	Left elbow	Right knee	Left knee	Right ankle	Left ankle		
A	20	0	0	0	0	0	0	0	A
B	23	0	0	0	0	0	0	0	A
B	17	0	0	0	0	0	0	0	A
B	7	0	0	0	0	0	0	0	A
A	21	0	0	0	0	2	0	2	A
A	11	0	0	0	0	3	3	6	A
A	11	0	0	0	0	0	0	0	A
A	19	0	0	1	1	0	0	2	A
B	38	0	0	0	11	0	0	11	B
A	28	0	0	2	0	12	7	21	B
A	29	2	2	0	0	7	7	18	B
B	26	5	5	5	0	0	0	15	B
A	26	6	6	5	5	4	0	26	B
B	28	6	0	0	0	6	6	18	B
A	31	7	7	0	0	7	0	21	B
A	54	9	13	9	13	0	7	51	B
A	35	10	10	0	0	10	10	40	B
A	44	11	11	12	12	13	13	72	B
A	35	6	6	6	6	6	6	36	B
A	45	11	12	0	12	0	0	35	B

Pettersson score A: patients with a score <5 for each joint; Pettersson score B: patients with a score ≥5 for any joint.
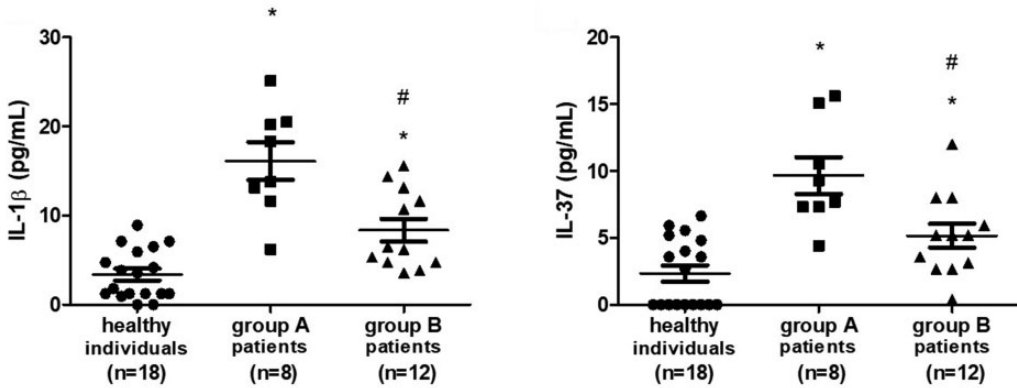
Figure 2. Analysis of interleukin (IL)-1β and IL-37 plasma levels in patients with different Pettersson score (indicating severity of haemophilic arthropathy) profiles. Plasma levels of IL-1β and IL-37 were significantly higher in Pettersson group A or group B patients than in healthy individuals and significantly lower in Pettersson group B patients than in Pettersson group A patients. *$p < 0.05$ compared with healthy individuals; #$p < 0.05$ compared with Pettersson group A patients. Group A represents patients with a Pettersson score <5 for all joints; Group B represents patients with a Pettersson score $\geq$5 for any joint.
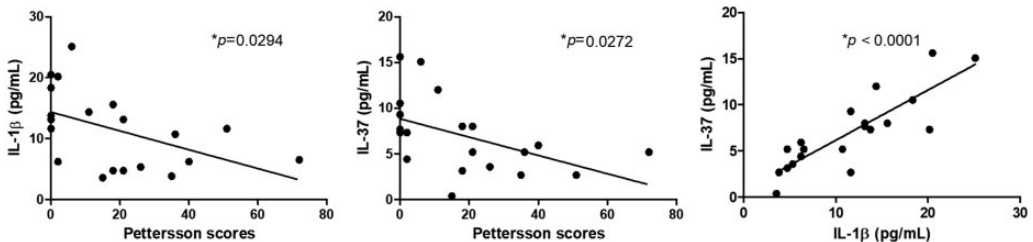

Figure 3. Correlation between cytokine levels and Pettersson score (indicating severity of haemophilic arthropathy). We found negative correlations between interleukin (IL)-1β ($r^2 = 0.2374$; $p = 0.0294$) and IL-37 ($r^2 = 0.2431$; $p = 0.0272$) plasma levels and the sum of Pettersson scores. We found a positive correlation between IL-1β and IL-37 plasma levels ($r^2 = 0.7196$; $p < 0.0001$). The sum of Pettersson scores represents the total scores of six joints (bilateral elbows, bilateral ankles, and bilateral knees).

joints. There was a negative correlation between IL-1β ($p = 0.0294$) and IL-37 ($p = 0.0272$) plasma levels and the total Pettersson scores and a positive correlation ($p < 0.0001$) between IL-1β and IL-37 plasma levels (Figure 3).

Discussion

HA is considered to result from the direct effects of blood components on cartilage and indirectly from synovial inflammation. Pro-inflammatory cytokines from

blood induce chondrocyte apoptosis and produce cartilage-degrading proteases.[10,26] Recombinant human IL-1β monoclonal antibodies or IL-1 receptor antagonists protect cartilage from blood-induced damage.[10] Although more in vivo studies and clinical trials are required, IL-1β has been shown to play a critical role in HA.

IL-37 is regulated by inflammatory stimuli and pro-cytokines through various signal transduction pathways. IL-37 is expressed and exerts anti-inflammatory

effects in various diseases, including RA, OA, melanoma, morbid obesity, contact hypersensitivity, atopic dermatitis, liver inflammatory injury, and systemic lupus erythematosus.[18,20,27] IL-37 regulates the inflammatory response mainly by inhibiting the expression, production, and functionality of pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6, IL-12, and TNF-α.[18] The silencing of IL-37 enhances cytokine production associated with pro-inflammatory stimuli.[18,22] An elevated IL-37 level has also been observed in patients with RA and erosive inflammatory OA and is positively correlated with disease activity.[14,16,20]

In this study, plasma levels of IL-1β and IL-37 were significantly higher in patients with severe haemophilia than in healthy individuals. The IL-37 plasma level was similar to the IL-1β expression profile and there was a positive correlation between IL-1β and IL-37 plasma levels in patients with severe haemophilia. This result may represent secretion of IL-37 to fight the pro-inflammatory response caused by IL-1β. In addition, IL-1β and IL-37 plasma levels were significantly lower in haemophilia patients with moderate to severe HA (group B, comprising those with Pettersson score ≥ 5 for any joint) than in those with no or mild HA (group A, comprising those with Pettersson score < 5 for all joints). The clinical stages in the developmental process of HA are recurrent bleeding, panarthritis, and fibrosis.[28] Bleeding and the severe inflammatory response usually occur at an early stage; bleeding is an uncommon manifestation in the late stage.[28] This may be why IL-1β and IL-37 plasma levels were lower in group B patients (closer to the fibrosis stage) than in group A patients (in the recurrent bleeding and panarthritis stages).

Patients in Pettersson group A were younger than patients in Pettersson group B (Table 1); however, we found no correlation between age and IL-1β ($p = 0.3774$) or IL-37 ($p = 0.9353$) plasma level in healthy individuals (data not shown). Therefore, we can exclude an effect of age on changes in IL-1β and IL-37 levels. HA is clinically less severe in patients with haemophilia B than in patients with haemophilia A.[29] However, we collected samples from patients with severe haemophilia A and severe haemophilia B in this study. In addition, our patients were grouped according to Pettersson score (the severity indicator of HA), which excludes the interference of haemophilia types. Although more research data are needed, IL-1β and IL-37 may be potential predictors of HA progression; that is, levels of IL-1β and IL-37 increase in the recurrent bleeding and panarthritis stages and then begin to decrease from the highest point, which may indicate that HA is progressing to the fibrosis stage.

Conclusions

Our results showed that plasma levels of IL-1β and IL-37 were significantly higher in patients with severe haemophilia than in healthy individuals and significantly lower in patients with moderate to severe HA than in those with no or mild HA. Continuous tracking of IL-1β and IL-37 plasma levels in patients with severe haemophilia could enable prediction of HA progression. Whether recombinant IL-37 can inhibit inflammation by inhibiting IL-1β signalling and has potential as a therapeutic agent for HA is worthy of further research.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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