J Gastroenterol 2006; 41:540–546
DOI 10.1007/s00535-006-1797-y

Journal of
Gastroenterology

Modulation of platelet aggregation responses by leukocytapheresis therapy in patients with active ulcerative colitis

Yuhki Yagi[1], Akira Andoh[1], Osamu Inatomi[1], Shigeki Bamba[1], Tomoyuki Tsujikawa[1], Yoshihide Fujiyama[1], Keiichi Mitsuyama[3], and Takashi Yoshida[2]

[1] Department of Internal Medicine, Shiga University of Medical Science, Seta Tukinowa, Otsu 520-2192, Japan
[2] Department of Clinical Laboratory Medicine, Shiga University of Medical Science, Otsu, Japan
[3] Second Department of Internal Medicine, Kurume University School of Medicine, Kurume, Japan

Background. Recent studies suggest that platelet activation plays an important role in the pathophysiology of inflammatory bowel disease. In this study, we evaluated the effects of leukocytapheresis (LCAP) on platelet functions in patients with ulcerative colitis (UC). ***Methods.*** Thirteen patients with active UC (five women and eight men) were treated with LCAP therapy. Platelet-rich plasma (PRP) was prepared, and platelet aggregation in response to agonist solution (epinephrine, collagen, and ADP) was measured with a platelet aggregometer. Platelet-derived microparticle (PDMP) plasma levels were determined by enzyme-linked immunosorbent assay. ***Results.*** Nine patients responded to LCAP therapy, but no clinical responses were observed in four patients. The aggregation response to 0.1 μg/ml epinephrine was enhanced in all patients. In all responders, enhanced epinephrine aggregation was normalized after the LCAP session. However, in the four nonresponders, enhanced epinephrine aggregation was maintained after the LCAP session. In responders, the mean maximum aggregation induced by 0.1 μg/ml epinephrine was 76.8 ± 5.0% before and 15.4 ± 3.8% after LCAP, respectively ($P < 0.05$). Increased aggregation responses to both 0.2 μg/ml collagen and 1.0 μM ADP were observed, and LCAP also normalized these enhanced responses. LCAP significantly reduced circulating PDMP levels (56.8 ± 28.3 U/ml before and 46.3 ± 30.4 U/ml after LCAP, $P < 0.05$). ***Conclusions.*** LCAP reduced enhanced platelet aggregation responses in active UC patients. Because platelets play an important role in inflammatory and immune responses, therapeutic effects of LCAP may be partially mediated by reduction of increased platelet aggregation activities.

Received: December 13, 2005 / Accepted: February 12, 2006
Reprint requests to: A. Andoh

Key words: IBD, inflammation, thrombosis

Introduction

The precise etiology of ulcerative colitis (UC) and Crohn's disease (CD), the two major forms of inflammatory bowel disease (IBD), remains unclear. Recent studies have suggested that mucosal inflammation in IBD is not dependent exclusively on dysregulated immune responses, but also involves the active participation of other cellular systems. Platelets are anuclear, nonimmune cells derived from the cytoplasm of bone marrow megakaryocytes, and play a key role in blood hemostasis and inflammation.[1,2] Recently, an increasing number of reports have cited the contribution of platelets to the pathogenesis of IBD. For example, an increase in the number of circulating platelets is a common feature of patients with active IBD.[3,4] A lower mean corpuscular volume of platelets is considered to be a potential marker of clinical activity in IBD,[5] and thromboembolic complications occur more frequently in patients with active IBD.[6,7] A strong activation of platelets in IBD patients is supported by the expression of platelet activation markers such as P-selectin and β-thromboglobulin.[8,9] Enhanced platelet activation in IBD is also supported by reports showing an elevation in various products such as platelet factor 4 and CD40 ligand.[8,10,11] Recently, we found that platelet aggregation responses to agonists (collagen, ADP, and epinephrine) are highly enhanced in patients with active IBD.[12] Furthermore, serum levels of platelet-derived microparticles (PDMPs), a new marker for platelet activation, are significantly elevated in patients with active IBD.[13]

Leukocytapheresis (LCAP) is a therapeutic strategy of extracorporeal immunomodulation that has been used to treat several immunological disorders, such as

rheumatoid arthritis.[14–17] In Japan, several open trials of LCAP in UC patients accumulated encouraging results in bringing steroid-resistant patients into remission.[18–22] For example, a multicenter randomized controlled trial reported by Sawada et al.[22] showed that the efficacy of LCAP therapy was significantly superior to that of high-dose steroid therapy (74% vs. 38%, $P = 0.005$). Furthermore, subjects who had undergone LCAP therapy had a lower incidence of adverse effects than those given high-dose steroid therapy.[20,22]

LCAP therapy for abnormal immune disorders aims to improve the conditions of the patient's pathology and to correct imbalances in immunological regulatory mechanisms by removing activated lymphocytes and monocytes/macrophages from the peripheral blood.[14] We recently demonstrated that LCAP significantly suppresses proinflammatory cytokine secretion from peripheral blood mononuclear cells (PBMCs).[23] LCAP also induces a relative increase in both Th2 CD4 T cells and $CD4^+CD25^+$ regulatory T cells.[24]

Several studies have demonstrated that LCAP reduces the number of circulating platelets in peripheral blood.[23] However, the effect of LCAP on platelet function remains unclear. In this study, we evaluated the effect of LCAP on platelet function in IBD patients by detailed platelet aggregometry. We found that LCAP suppresses the increased aggregation response of platelets in patients with active UC, suggesting that improvements in activated platelet functions may be involved in mechanisms underlying the therapeutic effects of LCAP therapy.

Methods

Patients

Thirteen in- and outpatients with UC (five women and eight men) and 20 healthy control subjects (ten women and ten men; mean age, 30.5 ± 6.5) were studied (Table 1). All patients were managed at the Division of Gastroenterology at the Shiga University of Medical Science Hospital. Study participants had diagnoses established by the usual radiological, histological, and clinical criteria. All patients had a high clinical activity index (CAI ≥ 5), as described by Rachmilewitz,[25] and were regarded as active-phase patients.

Table 1. Background factors for the 13 patients enrolled in this study

Demography	Measurement
Male/female	8/5
Age (years) (range)	33.5 ± 9.6 (20–52)
Duration of UC (years) (range)	4.5 ± 3.8 (0.5–11)
Number of relapses	3.4 ± 3.9 (1–8)
Classification of severity	
Severe	4
Moderate	9
Mean CAI[a] (range)	9.8 (7–17)
Extent of UC	
Total colitis	9
Left-sided colitis	4

UC, ulcerative colitis; CAI, clinical activity index
[a] According to Rachmilewitz,[25] the disease of some of these 13 patients was steroid dependent and that of others was steroid refractory

The ethics committee of Shiga University of Medical Science approved this project, and the blood was sampled after informed consent was obtained.

LCAP therapy

LCAP therapy was performed with a Cellsorba EX leukocyte removal filter (Asahi Medical, Tokyo, Japan).[22] Access and return lines were connected to bilateral cubital veins. A total of 3000 ml of whole blood was processed at a blood flow rate of 50 ml/min. Nafamostat mesilate (Torii Pharmaceutical, Tokyo, Japan) was used as an anticoagulant for the extracorporeal circulation. Drug administration was not altered during LCAP therapy. Each patient was treated by an LCAP session once per week, and treatment continued for 5 to 10 weeks. Each patient received a total of five to ten LCAP sessions. Clinical remission was defined as a decrease in the CAI to 4 or less. Patients who entered remission during one course of LCAP therapy were regarded as responders. Patients, whose CAI decreased to at least 5 points but remained above 4 were also considered responders. The clinical efficacy was determined after the fifth LCAP session. Nine patients were regarded as responders, while four patients were classified as nonresponders.

Sample collection and platelet aggregation tests

To minimize any platelet activation during sample collection, all blood samples were collected within 5 min just before and just after LCAP from the sampling port of the access line, which was located upstream of the anticoagulant connection. Samples were obtained at the first LCAP session. Conventional routine laboratory tests were conducted, and a complete blood count was performed. Blood samples were mixed with a one-tenth volume solution of ACD (2.2 g trisodium citrate, 0.807 g citric acid, 2.2 g dextrose in 100 ml of distilled water). Platelet-rich plasma (PRP) was prepared by centrifugation at 150 *g* for 5 min at room temperature. The PRP was added to a one-half volume solution of 0.1% EDTA/saline, and centrifuged at 1500 *g* for 20 min (platelet-poor plasma; PPP).

The number of platelets in the PRP was adjusted to 30×10^4/μl using PPP. A 200-μl aliquot of PRP and a magnetic stir bar was added to each channel of the

platelet aggregometer. Next, freshly prepared agonist solution (epinephrine, 0.1, 0.2, and 2.0 μg/ml; collagen, 0.2, 0.5, and 2.0 μg/ml; and ADP, 1.0 and 3.0 μM) was added. Platelet aggregation was then measured by a turbidometric method. Light transmission of the PPP was defined as the 100% aggregation value, and the light transmission before the addition of the agonist was regarded as 0% aggregation.

Measurement of PDMPs

PDMP levels were determined by an enzyme-linked immunosorbent assay system, as recently reported by Osumi et al.[26] This system is commercially available from Japan Immunoresearch Laboratories (Takasaki, Japan). One unit per milliliter of PDMP was determined to represent 2,400 solubilized platelets/ml.

Statistical analyses

Data are expressed as means ± SD. Comparisons between the means were performed using the Mann-Whitney U test. Differences resulting in P values less than 0.05 were considered to be statistically significant.

Results

Changes in complete blood counts

The average number of each blood cell type is shown in Table 2. The LCAP significantly reduced the number of white blood cells, platelets, monocytes, and lymphocytes.

Aggregation patterns

Normal patterns of platelet aggregation are shown in Fig. 1. In normal subjects, platelet aggregation was induced by a high concentration of agonist (2.0 μg/ml epinephrine, 2.0 μg/ml collagen, or 3.0 μM ADP), but the platelets did not respond to a low concentration of agonist (0.1 μg/ml epinephrine, 0.2 μg/ml collagen, or 1.0 μM ADP) (Fig. 1). Platelet aggregation caused by a low concentration of agonist represents an increased sensitivity of the platelets. In normal subjects, the maximum aggregation response to 0.1 μg/ml epinephrine, 0.2 μg/ml collagen, or 1.0 μM ADP was within 20%, 20%, or 40%, respectively. In the 20 healthy control subjects enrolled in this study, the mean maximum aggregation induced by 0.1 μg/ml epinephrine, 0.2 μg/ml collagen, and 1.0 μM ADP was 10.8 ± 2.2%, 10.4 ± 2.0%, and 27.5 ± 4.4%, respectively.

Changes in platelet aggregation responses

Effects of LCAP sessions on platelet aggregation responses in two representative cases (responder and nonresponder) are compared in Fig. 2.

The responder was a 32-year-old man. To maintain his remission status, he was on an oral dose regimen of 15 to 20 mg/day oral prednisolone (PSL) in combination with 2.25 g/day 5-aminosalicylic acid (5-ASA). He experienced a UC flare-up (CAI score, 10 points), and received LCAP therapy. In this patient, platelet responses to both 0.1 μg/ml epinephrine and 1.0 μM ADP were enhanced (Fig. 2, upper panels). After a LCAP session, these responses improved, resembling normal patterns.

The nonresponder was an 18-year-old man, who had been on 2.25 g/day 5-ASA to maintain his remission status. He experienced a UC flare-up (CAI score, 12 points), and received 50 mg/day oral PSL, followed by LCAP therapy. In this patient, enhanced aggregation responses to 0.1 μg/ml epinephrine, 0.2 μg/ml collagen, and 1.0 μM ADP were maintained following an LCAP session (Fig. 2, lower panels).

In the 13 UC patients, aggregation response maxima, induced by 0.1 μg/ml epinephrine, 0.2 μg/ml collagen, or 1.0 μM ADP, were compared before and after the first LCAP session. As shown in Table 3, aggregation responses to 0.1 μg/ml epinephrine were elevated in all responders, and significantly reduced upon LCAP (before, 76.8 ± 5.0%; after, 15.4 ± 3.8%). In the four nonresponders, platelet aggregation responses were also enhanced, and they remained elevated after the LCAP session (before, 80.3 ± 2.1%; after, 75.5 ± 2.1%).

Collagen aggregation was enhanced in six of nine responders and in all nonresponders (Table 3). In five of six enhanced responders, collagen aggregation returned to a normal range after the LCAP session, but in three of four nonresponders collagen aggrega-tion remained enhanced. Similarly, ADP aggregation was enhanced in seven of nine responders and in all nonresponders. Enhanced ADP aggregation was suppressed in six of seven enhanced responders, but remained at increased levels in three of four nonresponders.

Table 2. Changes in blood cell counts

	Pre-LCAP	Post-LCAP
Red blood cells ($\times 10^4$/μl)	411 ± 69	399 ± 58
Platelets ($\times 10^4$/μl)	27.0 ± 7.8	14.2 ± 3.2**
White blood cells (/μl)	8800 ± 3540	7650 ± 3200
Granulocytes (/μl)	7200 ± 2800	6300 ± 3800
Monocytes (/μl)	520 ± 250	320 ± 220*
Lymphocytes (/μl)	1250 ± 580	780 ± 370**

LCAP, leukocytapheresis
* $P < 0.05$
** $P < 0.01$

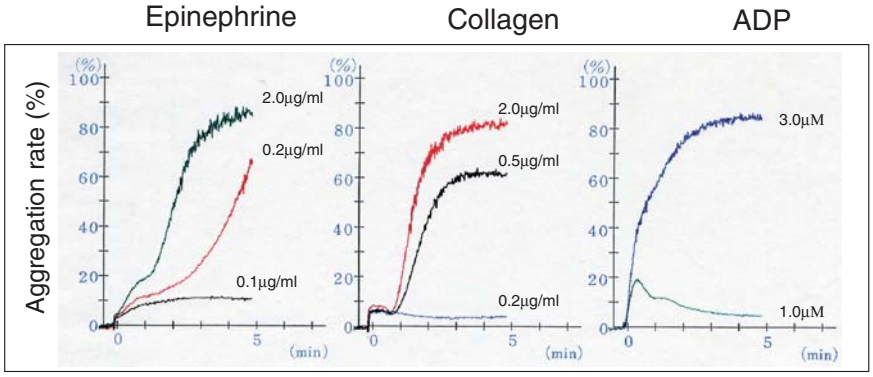
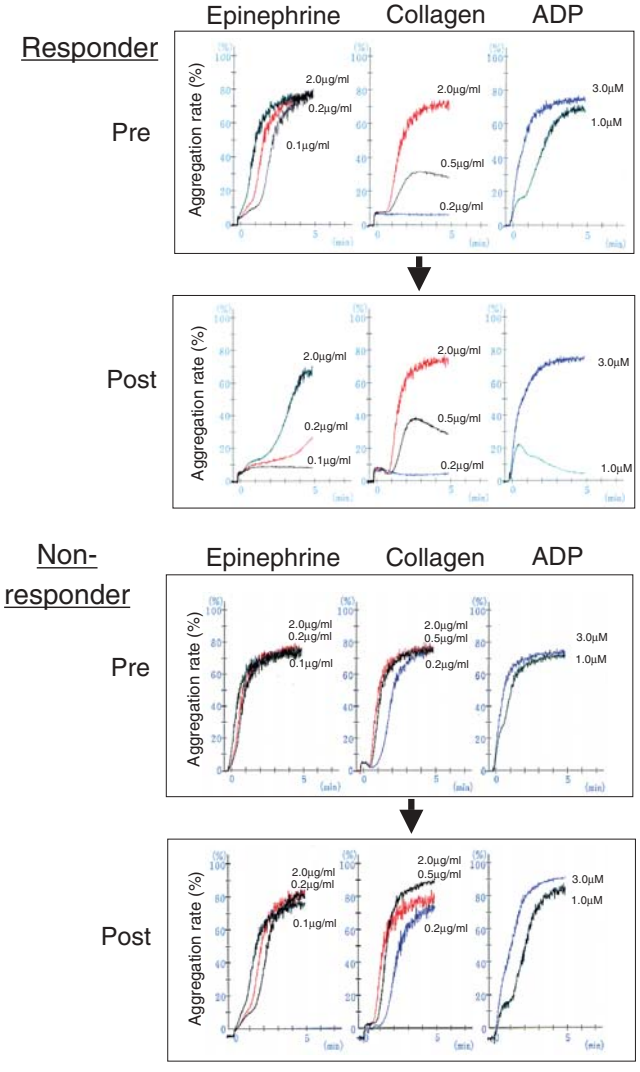


Fig. 1. Normal platelet aggregation patterns. The number of platelets in platelet-rich plasma (PRP) was adjusted to $30 \times 10^4/\mu l$ using platelet-poor plasma (PPP). A 200-μl aliquot of PRP was added to each channel of the platelet aggregometer, and agonist solutions (*epinephrine*, 0.1, 0.2, and 2.0 μg/ml; *collagen*, 0.2, 0.5, and 2.0 μg/ml; and *ADP*, 1.0 and 3.0 μM) were added. Platelet aggregation was measured by a turbidometric method. Light transmission of the PPP was regarded as 100% aggregation, and light transmission before the addition of the agonist was regarded as 0% aggregation



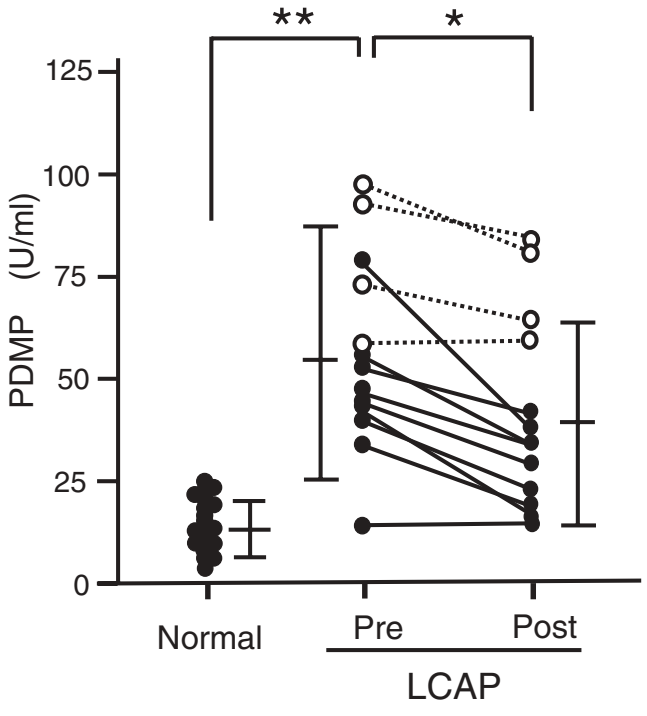


Fig. 3. Changes in circulating platelet derived-microparticle (*PDMP*) levels. *Closed circles* represent responders, and *open circles* represent nonresponders. *Bars* indicate means ± SD. *LCAP*, leukocytapheresis (**$P < 0.01$, *$P < 0.05$)

Fig. 2. Changes in platelet aggregation patterns in patients with active ulcerative colitis. In responders (*upper panels*), elevated aggregation responses were reduced after the LCAP session. In nonresponders (*lower panels*), aggregation responses remained elevated

Table 3. Changes in maximum platelet aggregation by LCAP therapy (%)

	Epinephrine (0.1 μg/ml)		Collagen (0.2 μg/ml)		ADP (1.0 μM)	
Case No.	Pre	Post	Pre	Post	Pre	Post
Responders						
1	81	15	14	8	72	22
2	78	18	10	10	76	32
3	72	12	12	10	26	32
4	78	12	74	12	72	22
5	82	16	72	12	74	30
6	84	24	72	12	78	22
7	72	16	76	10	78	76
8	74	14	74	72	80	22
9	70	12	76	14	26	24
Mean ± SD	76.8 ± 5.0	15.4 ± 3.8*	53.3 ± 31.0	17.8 ± 20.4*	64.7 ± 22.1	31.3 ± 17.3*
Nonresponders						
1	81	78	78	78	74	82
2	86	76	80	78	82	32
3	78	75	82	16	78	76
4	76	73	78	76	80	80
Mean ± SD	80.3 ± 2.1	75.5 ± 2.1	79.5 ± 1.9	62.0 ± 30.7	78.5 ± 3.4	67.5 ± 23.8

Underlined values indicate enhanced aggregation responses
* $P < 0.05$ versus pre-LCAP value

Effects of LCAP on circulating platelet-derived microparticles

Recently, we reported that circulating PDMPs, a novel marker of platelet activation, were elevated in active IBD patients.[13] As shown in Fig. 3, circulating PDMP levels were significantly higher in active UC patients than in normal individuals. Following LCAP sessions, circulating PDMP levels were significantly reduced in nine responders (before, 42.7 ± 14.2 U/ml; after, 27.7 ± 12.3 U/ml, $*P < 0.05$). However, in the four non-responders, circulating PDMPs persisted at high levels (before, 88.8 ± 26.6 U/ml; after, 78.0 ± 23.8 U/ml).

Discussion

Recent studies have revealed that platelets play an active role in a variety of inflammatory and immune processes.[2] Platelets store various chemokines of CXC and CC classes in α-granules, and release them upon activation.[1,2] Activated platelets express CD40 ligands (CD40L), and the binding of CD40-expressing immune cells such as B cells and monocytes triggers multiple inflammatory and immune responses.[2] Recently, Danese et al.[10,11] demonstrated that CD40-expressing IBD platelets release profuse amounts of chemokines when in contact with microvascular endothelial cells. Platelets also activate various immune and nonimmune cells through the release of various active mediators such as interleukin (IL)-1β, transforming growth factor, and platelet-derived growth factor.[2] Thus, platelets play an important role in both immune and inflammatory responses, and may be a possible target for therapeutic strategy in UC patients.

Various mechanisms by which LCAP exerts clinical effects on UC patients have been proposed. Kohgo et al.[27] reported that leukocytapheresis reduced the expression of adhesion molecules such as selectins and integrins on circulating PBMCs. Sawada et al.[18] also reported that LCAP reduced the percentage of activated T cells in peripheral blood, suggesting that removal of effector T cells from the circulation may decrease colonic inflammation. Noguchi et al.[28] reported that filter-passed PBMCs during LCAP therapy generated higher amounts of IL-4 than prefiltered PBMCs, suggesting that IL-4-secreting PBMCs might exert bystander suppressive effects in the inflamed mucosa. Hidaka et al.[17] evaluated serum cytokine levels before and after LCAP in rheumatoid arthritis (RA) patients. They found that LCAP significantly decreased serum tumor necrosis factor-α, IL-15, and RANTES levels and increased anti-inflammatory IL-10 levels. Recently, our group[23] and Mitsuyama et al.[29] showed that LCAP induces a PBMC hyporesponse to stimuli of inflammatory cytokines and lipopolysaccharides through inactivation of transcription factor NF-κB. LCAP has been reported to modulate peripheral T-cell subgroups and to induce elevation of Th2/Th1 ratio and $CD4^+CD25^+$ regulatory T cells.[24] Thus, potential mechanisms responsible for the anti-inflammatory effects of LCAP have been proposed from various standpoints.

However, the literature does not report how LCAP modulates platelet function.

Platelets respond to a number of stimuli by changing their shape from discoid cells to spherical spiny cells and by aggregating into large clumps. Experimentally, these dynamic processes can be measured by turbidometric aggregometry.[30] As the platelet suspension is stirred, agonists (e.g., collagen, ADP, and epinephrine) are added, and the coalescence of individual platelets into aggregates is measured by changes in light transmission.[30] This is a typical tool for the functional analysis of platelets, whereas various other markers such as sP-selectin, PF4, and β-TG are not functional but represent a consequence of platelet activation. The most characteristic feature of platelet aggregometry is its ability to serve as a functional evaluation tool of platelet samples. By means of this method, we found that platelet aggregation activity is enhanced in patients with active UC. Furthermore, activated platelet responses are improved by LCAP. Previous studies indicated that LCAP significantly reduces the circulating number of platelets in UC patients.[23] Actually, we observed that the number of platelets was significantly reduced following a LCAP session. Thus, LCAP reduces platelet numbers and improves enhanced aggregation responses in platelets, suggesting that LCAP exerts its anti-inflammatory effects through a reduction in platelet number and by reducing platelet functioning.

It remains unclear how anticoagulants (i.e., nafamostat mesilate) affect platelet functions during LCAP sessions. Our preliminary study of granulocyte/monocytes adsorptive apheresis therapy (GCAP), which also uses nafamostat mesilate as an anticoagulant, showed that GCAP does not modulate enhanced platelet functions in active UC patients. The aforementioned result indicates that anticoagulants do not affect the functioning of circulating platelets during GCAP sessions, and indirectly suggests that the modulation of platelet functions by LCAP may not be associated with anticoagulant effects.

Four patients did not respond to the LCAP therapy. In nonresponders, improvements in increased platelet aggregation responses were not observed. In previous studies, several predictive factors for a positive response to LCAP therapy have been reported. Sawada et al.[18] showed that the percentage of activated T cells was elevated in responder UC patients, but the T-cell percentage was within the normal range in nonresponders. Hidaka et al.[17] observed a decrease in serum IL-15, GM-CSF, and RANTES levels only in responders, but not in nonresponders. Given the possibility that platelets play an important role in the pathophysiology of UC, reduction of the platelet aggregation response may be one of several predictive factors for a positive response to LCAP therapy. Since the number of patients in the present study was small, greater numbers of UC patients need to be evaluated in order to determine suitable predictive factors.

Recently, we reported that PDMPs are novel markers of platelet activation in IBD patients.[13] The generation of PDMPs is associated with platelet activation induced by stimuli such as high shear stress, thrombin, collagen, and calcium ions.[31,32] PDMPs play a role in hemostatic responses under normal and pathological conditions.[32] In addition, previous studies have reported a role for PDMPs during inflammatory responses.[32,33] As shown in Fig. 3, PDMP levels are significantly elevated in patients with active UC, and this finding is compatible with the results of our recent study showing that circulating PDMP levels correlate with disease activity in IBD patients. Interestingly, LCAP significantly reduces circulating PDMP levels in these patients. Since PDMPs are associated with platelet activation, the aforementioned result suggests that LCAP actually improves platelet functioning and reduces platelet activation in the body.

In conclusion, we demonstrated that LCAP suppresses enhanced platelet aggregation in active UC patients. Since recent studies have demonstrated that platelets play a role not only in hemostasis but also in inflammatory and immune responses, the anti-inflammatory effect of LCAP may be partially mediated by reductions in increased platelet aggregation activities.

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