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Original Article

Circulating platelet–leukocyte aggregates in patients with inflammatory bowel disease

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Abstract

Background: Inflammatory bowel diseases (IBDs), Crohn's disease, and ulcerative colitis are considered to be chronic inflammatory disorders implicated with recurrent tissue damage to the intestine. There is a positive correlation between platelet–leukocyte aggregates and ischemic vascular risk. There are limited data about the relationship between platelet–leukocyte aggregates and IBD. This study was designed to determine whether platelet–leukocyte aggregates increase in IBD, and whether a relationship exists between the elevation of platelet–leukocyte aggregates and disease activity.

Methods: A total of 20 patients with IBD (16 with ulcerative colitis and 4 with Crohn's disease) and 20 healthy controls participated in our study. Nine patients were in active-phase IBD, whereas 11 patients were in inactive phase. To show the presence of thrombocyte aggregates, the monoclonal antibodies such as Isotype IgG1 mouse antihuman CD42b-PE (phycoerythrin) (Beckman Coulter IMI417), Isotype IgG1 mouse antihuman CD45-FITC (fluorescein isothiocyanate) (Beckman Coulter IM0782), and Isotype IgG2a mouse antihuman CD45RO-FITC (Beckman Coulter IMI247) were used. Additionally, the values of platelet–neutrophil aggregates were measured in peripheral blood samples using flow cytometry techniques.

Results: The levels of platelet–leukocyte aggregates in blood samples were found to be significantly higher during both the active and inactive phases in patients with IBD. There were no statistically significant differences between active-phase and inactive-phase patients.

Conclusion: We determined that the patient group had significantly higher platelet–leukocyte aggregate levels compared with the control group. This finding suggests that platelet–leukocyte aggregates may play a role in the development of IBD.

Keywords: disease activity; inflammatory bowel disease; platelet–leukocyte aggregate

1. Introduction

Inflammatory bowel diseases (IBDs), Crohn's disease and ulcerative colitis, are considered chronic inflammatory disorders mediated by the immune response of the host and implicated with recurrent tissue damage to the intestine. To date, their pathogenesis has not yet been precisely determined.[1]

During the activation phase of the disease in young patients, spontaneous thromboses can occur on different sites of the body. Additionally, this exacerbation is accompanied by thrombocytosis, which increases the risk of a thromboembolic event.[2] Various hemostatic pathologies were determined in patients with IBD such as thrombocytosis, a decrease in anticoagulant activities of antitrombin-3, protein-S, and tissue plasminogen activator, an increase in the levels of fibrinogen, plasma fibrinopeptid-A and factor 8, as well as an increase in monocyte procoagulant activity. These features suggest the presence of an underlying vascular inflammatory and prothrombotic process.[3]

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There is a positive correlation between platelet−leukocyte aggregates (PLAs) and ischemic vascular risk.[4] The results of previous studies suggest that PLAs are more active than circulating leukocytes alone.[5−7] Leukocytes and platelets play significant roles in the pathogenesis of IBD. Therefore, it is reasonable to hypothesize that PLAs might play a role in IBD, which is an inflammatory disorder.

P-selectin is located on platelet surfaces and has a key role in the formation of PLAs. In addition to P-selectin, CD42b and CD45 are also involved in this process. CD42b is the alpha subunit of glycoprotein Ib. The binding of the glycoprotein Ib−IX−V complex in conjunction with the von Willebrand factor facilitates initial platelet adhesion to vascular subendothelium after vascular injury, and also initiates signaling events within the platelet that lead to enhanced platelet activation, thrombosis, and hemostasis. CD45 is a family of single-chain transmembraneous glycoproteins and are located in all leukocytes. CD45 is also called the common leukocyte antigen, protein tyrosine phosphatase receptor type C. Antibodies recognizing a common epitope on all of the isoforms are termed CD45, whereas those recognizing only individual isoforms are termed CD45RA or CD45RO, etc. CD45RO is located in memory T cells, and facilitates T cell activation.

There are limited data about the relationship between PLAs and IBD. In our study, we measured the values of platelet−neutrophil aggregates in peripheral blood samples using flow cytometry techniques in patients with IBD.

2. Methods

Our study included 20 patients whose follow-up took place at the Istanbul Private Memorial Hospital. Among these patients, four had Crohn's disease (mean age, 54.5 years) whereas 16 had ulcerative colitis (mean age, 43.8 years). When data from the two groups are combined, patient age varied between 28 and 65 years, and mean age was 41.5 years.

Nine patients were in the active phase, whereas 11 patients were in the inactive phase. For patients with Crohn's disease, clinical activity was measured with the Crohn's disease activity index, and that in patients with ulcerative colitis was measured using the Rachmilewitz endoscopic index. Crohn's disease patients with a Best activity index score >150[8] and ulcerative colitis patient with a Rachmilewitz score ≥ 5 were considered to have an active disease.[9]

All of the active-phase patients had ulcerative colitis. Twenty age- and sex-matched healthy voluntary individuals served as the control group (mean age, 39.8). Patients with a history of peripheral vascular disease, hypertension, cardiopulmonary bypass, coronary angioplasty, chronic obstructive pulmonary disease, hemodialysis, diabetes mellitus, or renal insufficiency were excluded. Any patient and control group member who had taken any drug treatment including acetylsalicylic acid or nonsteroid anti-inflammatory drug, or who smoked were also excluded. This study was approved by the hospital administration of the Istanbul Private Memorial Hospital. Study participants provided signed informed consent.

2.1. Blood sampling

Blood samples were drawn from patients through a vein in the region without stasis, and then passed into the siliconized vacutainer tubes, which contained 3.6 mg of sterilized K2E (Becton Dickinson).

2.2. Monoclonal antibodies

To show the presence of thrombocyte aggregates, the following monoclonal antibodies were used in our study: Isotype IgG1 mouse antihuman CD42b-PE (phycoerythrin) (Beckman Coulter IMI417), Isotype IgG1 mouse antihuman CD45-FITC (fluorescein isothiocyanate) (Beckman Coulter IM0782), and Isotype IgG2a mouse antihuman CD45RO-FITC (Beckman Coulter IMI247).

2.3. Flow-cytometric analysis

We used a Beckman Coulter EPICS ELITE analyzer for flow cytometric analysis. Each tube (CD42a/CD62P, CD42b/CD62P) was separately prepared for patient and control group blood samples and contained 20 μL of monoclonal antibody, which was subsequently mixed with 20 μL of blood sample, and finally incubated for 15 minutes at room temperature. Then, these tubes were processed by a Coulter Multi Q preparation device. Immunoprep A, B, and C solutions (Immunoprep A: formic acid−stabilizer; Immunoprep B: sodium carbonate−chloride−sulfate−stabilizer; and Immunoprep C: paraformaldehyde−buffers Coulter Immunoprep reagent system PN7507950-D) were then added. After 15 minutes, the samples were ready for flow cytometric analysis. On the second dot plot, another gating was performed on the cell population that contained CD45 positive cells. In accordance with this gating process, the PLAs that existed in the marked area of CD45/CD42b, CD45RO/CD42b dot plot were displayed and then analyzed (Fig. 1).

2.4. Statistical analysis

We used a one-way ANOVA test with *post hoc* Bonferroni test to evaluate the investigative data. A p value of <0.05 was considered statistically significant.

3. Results

The levels of PLAs (CD45/CD42b and CD45RO/CD42b) were found to be significantly higher in the patient group with IBD than in the control group [mean value of $6.75 \pm 1.15\%$ (SEM) and $1.65 \pm 0.16\%$ for the patient group and the control group, respectively; $p = 0.0001$]. The PLA values of nine patients in the active phase of IBD were found to be significantly higher when compared with those of the control group (mean value of $8.15 \pm 1.92\%$ and $1.65 \pm 0.16\%$ for active-phase patients and the control group, respectively; $p = 0.0001$). Similarly, the PLA values of 11 inactive-phase patients were found to be significantly higher when

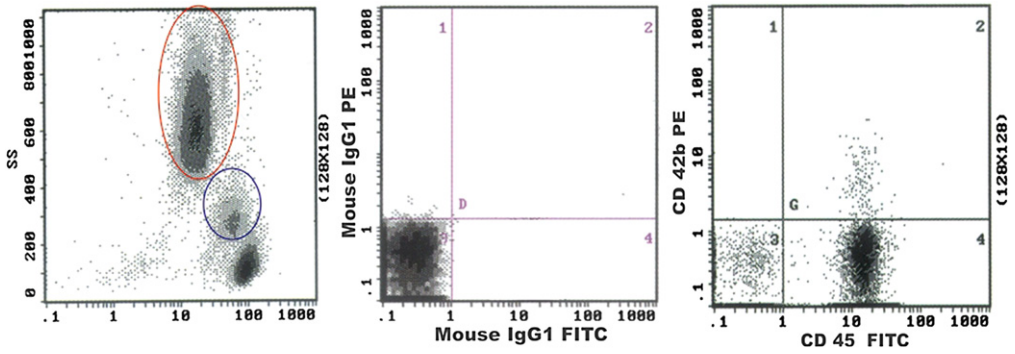

Fig. 1. Flow cytometric analysis for CD45 and CD42b.

compared with those of the control group (mean value of 4.56 ± 0.42% and 1.65 ± 0.16% for inactive-phase patients and the control group, respectively; $p = 0.0001$).

When the inactive-phase and active-phase patient groups were compared, the PLA values of the active-phase patient group (8.15 ± 1.92%) were higher than those of the inactive-phase patient group (4.56 ± 0.42%). The difference was found to be statistically significant ($p = 0.0001$).

4. Discussion

Using flow-cytometric techniques, we found that the values of PLAs were significantly higher in the patient group than in the control group.

After initiating the neutrophil chemotaxis process, the hyperaggregable active platelets in IBD may amplify the inflammatory cascade. The P-selectin (CD62P) released by the platelets facilitates the migration of neutrophils into the inflammation area. Other mediators released by the active platelets including platelet factor-4, platelet-activating factor, seratonin, and 12-hydroxyeicosatetraenoic acids show reactions as a chemotactic factor for neutrophils. In addition, platelet factor-4 stimulates the release of elastase from neutrophils. Active platelets can start the diapedesis process of neutrophil by stimulating IL-8 release from the endothelium. Circulating platelet aggregates may lead to the occlusion of intestinal microveins. Moreover, thromboxane A2 may contribute to the likelihood of ischemia after stimulating with local vasoconstriction.[10] The platelet aggregate values are higher in patients with IBD in peripheral circulation. It is stated that the cause of this elevation was platelet aggregation that started in the mesenteric vessels.[11]

During the process of inflammation, platelets bind to leukocytes to form PLAs. The first adhesion of platelets to neutrophils or monocytes occurs via the binding of P-selectin to its specific ligand on the leukocyte surface, P-selectin glycoprotein ligand-1. The stabilization of leukocyte–platelet aggregates occurs via the binding of leukocyte surface Mac-1 to platelet surface glycoprotein Ib (GPIb), integrin αIIbβ3, and/or junctional adhesion molecule 3. It is thought that an increased PLA value is a sensitive marker of inflammation and platelet activity.[12,13]

An increased number of PLAs occur in inflammatory states such as diabetes mellitus, cystic fibrosis, asthma, pre-eclampsia, nephrotic syndrome, sickle cell disease, and collagenous tissue diseases. Additional research is necessary to clarify the relationship between PLAs and IBD.

In our study, we determined that significantly higher PLAs existed in the patient group compared with the control group. This finding suggests that PLAs may be important in the pathogenesis of IBD.

We found that PLAs are significantly and statistically higher both in the active- and inactive-phase patient groups compared with the control group. When active- and inactive-phase patients were compared, no statistically significant difference was found. This situation reflects the fact that the abnormal hemostatic mechanism still prevailed, and patients had not completely recovered although they had shown indications of clinical remission.

Thus, it is reported that only 29% of patients in clinical remission had also achieved an endoscopic remission.[14]

Similar to our study, Irving et al[15] stated that the formation of PLAs was part of the increased platelet activation and played a role in IBD pathogenesis. The relationship between PLA formation and clinical and laboratory disease activity markers (platelet count, white blood cell count, activation of platelet and neutrophil) has not yet been established.[15] There had not been any definitive study done on this subject until Irving et al reported the results of their investigation. Our study is another example showing that PLAs play a role in IBD pathogenesis.

One of the limitations of our study is the sample size. However, when we excluded those patients with factors that raise PLAs, the sample size decreased. We have to emphasize only the PLA increase in IBD patients without any comorbidities. Formation of PLAs is increased in several inflammatory conditions. This may result from platelet and neutrophil activation, but may also contribute to the inflammatory process.[15] It is strongly believed that PLAs play a role in IBD pathogenesis; however, additional studies must be completed to evaluate whether the increase in PLAs are the result or cause of IBD.

Our results suggest that the agents that may prevent PLA formation or efficiency could actually be of use in the treatment of IBD. Accordingly, it was shown that administration of a recombinant soluble P-selectin glycoprotein ligand-1 preserved

vascular endothelial function.[16] When considering the difficulties associated with the treatment of IBD and the nature of such a disease, which is characterized by irregular disease progress and exacerbation, comprehensive studies of PLAs are needed in order to develop new therapeutic strategies.

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