Increased responsiveness to thrombin through protease-activated receptors (PAR)-1 and -4 in active Crohn's disease

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Abstract

Background and aims: Platelets are essential in hemostasis and inflammation, thereby linking coagulation with inflammation. Abundant thrombin generation in association with inflammation is considered a major reason for the increased risk for thromboembolic events. We therefore investigated platelet responsiveness to thrombin.

Methods: In this case–control study 85 patients with Crohn’s disease (active CD 42, remission 43) and 30 sex- and age-matched controls were enrolled. Clinical disease activity (Harvey–Bradshaw-Index) was assessed and CD-related data were determined by chart review. Platelets’ response to protease activated receptor-1 and -4 (PAR-1, -4) was assessed by whole blood platelet aggregometry (MEA), levels of platelets adhering to monocytes (PMA), and platelet surface P-selectin.

Results: Platelets’ aggregation after activation with the specific PAR-1 agonist (SFLLRN) and PAR-4 agonist (AYPGKF) was higher in patients with active CD compared to patients in remission and controls (p = 0.0068 and p = 0.0023 for SFLLRN, p = 0.0019 and 0.0003 for AYPGKF). Likewise, levels of PMA after activation with PAR-1 and PAR-4 receptor agonists were higher in patients with active CD compared to patients in remission and controls (p = 0.0001 and p < 0.0001 for SFLLRN, p = 0.0329 and p = 0.0125 for AYPGKF). However, P-selectin expression on human platelets showed heterogeneous results. Only PAR-1 activation of platelets resulted in significant differences between CD patients and controls (p = 0.0001 and p = 0.0022 for active and inactive CD versus controls, respectively).

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1. Introduction

The two major forms of inflammatory bowel disease (IBD), Crohn’s disease (CD) and ulcerative colitis (UC), result from complex interactions between evolving environmental changes, more than 150 of predisposing genetic mutations, a complex gut microbiota that may be continuously varied, the intricacies of individual immune systems and non-immune systems such as the hemostatic system. Among these, platelets are believed to play a crucial role as it is evident that, in addition to their role in primary hemostasis, platelets also play an active role in inflammatory processes. The concept of enhanced platelet activity in IBD is supported by reports showing significantly increased platelet aggregation in response to agonists like ADP, collagen, and ristocetin in both forms of IBD. While platelets are traditionally seen as the major players under high shear conditions like in atherosclerosis, there is growing evidence for their active role in venous thromboembolic disease. We propose that the clinical importance of increased platelet activation is reflected by a substantially increased incidence of thromboembolisms in IBD.

Continuous thrombin generation is considered a major reason for an increased risk of thromboembolic events, as it is a very potent platelet activator. Thrombin activates platelets via 4 distinct receptors, protease-activated receptors (PAR)-1, PAR-4, glycoprotein (GP) Ibα and GPV. PAR1 mediates platelet responses at low concentrations of thrombin while PAR4 mediates platelet activation only at high thrombin concentrations. PAR-1 and PAR-4 form a heterodimeric complex and with GP Ibα complementary units a trimeric receptor complex regulating platelet activation by thrombin, that enables thrombin to act as a bivalent or even trivalent functional agonist. Thus, coordinate activation of PAR by subnanomolar thrombin concentrations has been proposed. Clinically, there is strong evidence that PAR mediated platelet activation is significant. IBD is associated with altered plasma levels of a variety of hemostatic biomarkers indicating subclinical activation of the coagulation system, finally leading to enhanced generation of thrombin.

The complex interplay between inflammation and hemostasis results in activated platelets, aggregate formation, and release of P-selectin. The latter is the most important platelet "releasate" for the interaction with peripheral blood leukocytes, including monocytes, leading to enhanced leukocyte activation. High levels of platelet–monocyte aggregates (PMA) are regarded a very sensitive marker of thromboembolic disease. Indeed, increased levels of PMA have been reported in a variety of inflammatory diseases that are associated with an increased risk for thromboembolic disease, including IBD.

We assessed the association between inflammation in CD patients with their platelets' responsiveness to thrombin. We compared the responsiveness of platelets to two major thrombin receptors, PAR-1 and PAR-4, to their specific agonists (SFLLRN and AYPGKF for selectively activating PAR-1 and PAR-4, respectively) by platelet aggregation, determination of the formation of PMA, and levels of P-selectin expression of patients with active CD, patients in remission and controls. Thereby we show an enhanced responsiveness specific for PAR-inducible platelet activation in patients with active CD compared to patients in remission and controls. These findings may have the potential to explain the increased susceptibility for thrombin mediated risk of thromboembolic disease in patients with active CD.

2. Materials and methods

2.1. Patients and controls

The study complied with the Declaration of Helsinki, was approved by the Ethics Committee of the Medical University of Vienna, and all patients and healthy controls gave written informed consent. We enrolled 42 consecutive patients with active CD, 43 patients with CD in remission, and 30 age and sex-matched controls with coeliac disease under gluten-free diet without any symptoms as controls (Table 1). All participants were outpatients and recruited from the Department of Internal III, Division of Gastroenterology and Hepatology, Medical University of Vienna, Vienna, Austria. All CD patients were older than 18 years and had an established diagnosis of CD (based on clinical, endoscopic, histological, and radiological criteria according to European Crohn’s and Colitis Organisation (ECCO) guidelines). CD-related data were collected from chart review and included age at diagnosis, disease extent and phenotype, CD-related surgery, medication, smoking habits, and disease activity. Extent of CD and disease behavior were classified according to the Montreal classification. Active disease was defined by a Harvey-Bradshaw Index (HBI) of >4. CD-related surgery was defined as bowel resection only. A smoker was defined as a patient who smoked at least 7 cigarettes weekly for at least 1 year. The diagnosis of coeliac disease was based on typical morphology of duodenal mucosal biopsy specimens taken during oesophago-gastroduodenoscopy confirmed by positive antibodies, according to ESPGHAN criteria. Patients with coeliac disease in long-term remission served as controls. They were symptom-free on gluten-free diet for at least one year and were anti-endomysial antibodies (EMA) and anti-tissue-transglutaminase antibodies — negative at any time of the study. Exclusion criteria included hereditary platelet abnormalities, a family or personal history of bleeding disorders, significant renal dysfunction, malignant paraproteinemias, myeloproliferative disorders, severe hepatic failure, malignancies, patients with adipose phenotype (BMI > 30 kg/m2), a major surgical procedure within 3 months.
before enrolment, the administration of anti-platelet therapy, of aspirin or non-steroidal anti-inflammatory drugs, 5-aminosalicylic acid therapy, therapy with vitamin K antagonists (warfarin, phenprocoumon, acenocoumarol) in the previous 14 days, a platelet count \( \leq 100,000 \) or \( \geq 450,000/\mu l \) and a hematocrit \( \leq 30\% \).

### 2.2. Blood sampling

Blood was drawn by clean venipuncture from an antecubital vein using a 21-gauge butterfly needle (0.8 × 19 mm; Greiner Bio-One, Kremsmünster, Austria). Blood samples were taken by the same physician applying a light tourniquet as previously described, which was immediately released.40 Samples were mixed adequately by gently inverting the tubes. The initial 3 mL of blood was used for routine blood cell counts, and then blood was drawn into a 3.2% sodium citrate Vacuette tube (Greiner Bio-One; 9 parts of whole blood, 1 part of sodium citrate 0.109 M/L) for analyses by flow cytometry, while hirudin-anticoagulated blood (13 \( \mu g/mL \)) was used for the determinations by whole blood multiple electrode impedance aggregometry (MEA).

### 2.3. Multiple electrode aggregometry (MEA)

MEA (Dynabyte, Munich, Germany) was used to specifically determine the in vitro responsiveness of patients' platelets to SFLLRN (PAR-1 activation, 32 \( \mu M \); Bachem, Bubendorf, Switzerland) and AYPGKF (PAR-4 activation, 662 \( \mu M \); Dynabyte). The concentrations of agonists were used as suggested by the manufacturer. In brief, hirudin-anticoagulated whole blood was diluted 1:2 with 0.9% NaCl solution and stirred in the test cuvettes for 3 min at 37 °C. Then agonists were added and aggregation was continuously recorded for 5 min. Adhesion of activated platelets to the electrodes led to an increase of impedance, which was detected for each sensor unit separately and transformed to aggregation units (AU) that were plotted against time.

### 2.4. Determination of platelets adhering to monocytes (PMA)

Platelets adhering to monocytes were identified as previously published.41 In brief, agonists for PAR-1 (SFLLRN, 7.1 \( \mu M \); Bachem), PAR-4 (AYPGKF 357 \( \mu M \); Dynabyte), or...
HEPES buffer were added to 5 μl whole blood, diluted in 55 μl HEPES-buffered saline. After 15 min, monoclonal antibodies (anti-CD45, clone 2D1-peridinin chlorophyll protein labeled, Becton Dickinson, B.D., San Jose, CA; anti-CD41 clone P2, phycoerythrin labeled, Immunotech, Beckman Coulter Fullerton, CA, USA), and anti-CD14– (clone M0P9, alllophycocyanin labeled, B.D.), or isotype-matched controls were added. After 20 min, samples were diluted with FACS Lysing solution and 10,000 CD45+ events were acquired immediately. Within these events, lymphocytes, granulocytes, and monocytes were identified, based on their CD14 versus side scatter characteristics. Monocytes were identified as CD14+ and the CD45 + CD41 + and CD45 + CD41− events. Data are shown as gemoan fluorescence intensity (MFI). All samples were evaluated within 15 min after blood withdrawal. The agonists’ concentrations for the determination of PMA were determined in an independent cohort of 20 healthy individuals by titration experiments. Suboptimal concentrations were selected and resulted in at least a MFI of: PMA-SFLLRN 8 (median: 65, range: 8–292), PMA-AYPGKF 8 (median: 20, range: 8–292).

2.5. Regulation of P-selectin

Platelet activation was analyzed by P-selectin expression, without and after in vitro addition of the PAR-1 agonist SFLRN or the PAR-4 agonist AYPGKF. In brief, whole blood was diluted with HEPES buffer to obtain 20 × 10^9/L platelets. The expression of P-selectin was determined without agonists and after in vitro exposure to the PAR-1 agonist (SFLLRN, 5.7 μM; Bachem) or the PAR-4 agonist (AYPGKF, 714 μM; Dynabate). After 15 min, the platelet population was identified by staining with anti-CD42b (clone HIP1, alllophycocyanin labeled, BD), and P-selectin expression was determined by the binding of the monoclonal antibody anti-CD62p (clone CLB-Thromb/6, phycoerythrin-labeled, Immunotech). The reaction was stopped by adding 500 μl PBS, and samples were acquired immediately on a FACS Calibur flow cytometer (B.D.) with excitation by an argon laser at 488 nm and a red diode laser at 635 nm at a rate of 200–600 events per second. Standard B.D. Calibrate beads were used for daily calibration of the cytometer. Data are shown as gemoan fluorescence intensity (MFI). The agonists’ concentrations for the determination of P-selectin expression were determined in an independent cohort of 20 healthy individuals by titration experiments. Suboptimal concentrations were selected and resulted in at least a MFI of: P-selectin-SFLLRN 5 (median: 23, range: 5–109), P-selectin-AYPGKF 6 (median: 29, range: 6–250).

2.6. Statistical analyses

The primary endpoint was aggregation units measured by MEA in CD compared to controls. Considering a minimum expected difference (effect size) of 10 (AU controls = 95 vs. AU active CD = 105) the projected sample size at a level of 0.05 and a power of 0.8 was at least 30 per group (active CD, CD in remission, and control group). Secondary endpoints were levels of platelets adhering to monocytes and surface P-selectin expression. Data are presented as median with interquartile range (IQR), unless indicated otherwise. Platelet function data measured by MEA were normally distributed; therefore we performed t-tests to detect differences of platelet reactivity. Since the data of the secondary endpoints indicated a violation of the distributional assumptions for the t-test, evaluated by the D’Agostino & Pearson omnibus K2 test, the non-parametric Mann–Whitney-U test was used. Providing two-tailed significance levels, differences were considered statistically significant at p < 0.05. Statistical analysis was performed using SPSS version 17.0 (Chicago, IL, USA).

3. Results

3.1. Clinical and laboratory data

Clinical and laboratory characteristics of the study population are shown in Tables 1 and 2. Compared to controls and to CD in remission, platelet count, WBC count, fibrinogen, and CRP were significantly higher in active CD whereas transferrin saturation was significantly lower (Table 2).

3.2. Platelet reactivity by multiple electrode aggregometry

In patients with active CD SFLLRN and AYPGKF inducible platelet reactivity was significantly higher than in controls (p = 0.0023 for SFLLRN, Fig. 1a; p = 0.0003 for AYPGKF; Fig. 1b) as well as in CD patients in remission (p = 0.0068 for SFLLRN, Fig. 1a; p = 0.0019 for AYPGKF; Fig. 1b). No significant difference was observed between controls and controls.

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<thead>
<tr>
<th>Table 2</th>
<th>Laboratory data and markers of inflammation.</th>
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<td>Controls (n = 30)</td>
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<td>Laboratory data (median, IQR)</td>
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<tr>
<td>Platelet count (&lt;10^9/μl)</td>
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<td>CRP (nmol/L)</td>
<td>0.14 (0.05–0.28)</td>
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<td>WBC count (&lt;10^9/μl)</td>
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<tr>
<td>Fibrinogen (μmol/L)</td>
<td>311 (297–351)</td>
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<td>Transferrin saturation%</td>
<td>23.8 (18.3–30.6)</td>
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Values are median values (interquartile range).
^a p values are determined by the Mann–Whitney U test and are calculated from each patient group vs. the control group.
^b p values from CD subgroups (remission vs. active).

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controls (data not shown). The in vitro addition of agonists for PAR-1 and PAR-4, respectively, resulted in significantly higher levels of PMA in patients with active CD than in controls (p = 0.0001 for SFLLRN, Fig. 2a; p = 0.0125 for AYPGKF, Fig. 2b) and patients in remission (p = 0.0001 for SFLLRN, Fig. 2a; p = 0.0329 for AYPGKF, Fig. 2b). There was no difference between controls and CD in remission (p = 0.2868 for SFLLRN, Fig. 2a; p = 0.7120 for AYPGKF; Fig. 2b).

3.4. Regulation of P-selectin

Levels of P-selectin expression ex vivo (determined prior to exogenously added agonists) were not significantly different and CD patients in remission (p = 0.5788 for SFLLRN, Fig. 1a; p = 0.2761 for AYPGKF; Fig. 1b).

3.3. PMA formation

Levels of PMA ex vivo (determined prior to exogenously added agonists) were not significantly different in patients with active CD, compared to patients in remission and to

Figure 1  a, b: Platelet reactivity by multiple electrode aggregometry (MEA) in aggregation units (AU) in response to PAR-1 (SFLLRN) and PAR-4 agonist (AYPGKF) in inactive and active patients with Crohn’s disease and in controls (patients with coeliac disease under gluten-free diet). Boundaries of the box show the lower and upper quartile of data, the line inside the box represents the median. Whiskers are drawn from the edge of the box to the highest and lowest values that are outside the box but within 1.5 times the box length. Values outside this range (outliers) are shown as circles.

Figure 2  a, b: Levels of platelet–monocyte aggregates (PMA) after in vitro addition of PAR-1 (SFLLRN) or PAR-4 agonist (AYPGKF) in patients with inactive and active Crohn’s disease and from controls (patients with coeliac disease under gluten-free diet). Data are shown as geomean fluorescence intensity (MFI). Design as in Fig. 1.
in patients with active CD from controls or from those in remission or between patients in remission and controls (data not shown). Levels of P-selectin expression of PAR-1 activated platelets from patients with active CD were significantly higher than from controls ($p = 0.0001$; Fig. 3a). The corresponding data for patients with CD in remission were also significantly higher compared to controls ($p = 0.0022$; Fig. 3a), whereas no difference was observed between the CD subgroups ($p = 0.3294$; Fig. 3a). P-selectin expression of PAR-4 activated platelets from patients with active disease was not significantly different from platelets from CD in remission, or controls ($p = 0.5078$ and $p = 0.9582$; Fig. 3b).

4. Discussion

By studying PAR-1 and PAR-4 inducible platelet activations, we specifically addressed platelet responsiveness to two major thrombin receptors in CD patients. Our data indicate an increased susceptibility of platelets from patients with active CD for PAR inducible platelet activation resulting in increased aggregation and increased formation of platelets adhering to monocytes. These findings may explain the increased risk for thromboembolic events in patients with active CD.

Levels of thrombin, the strongest platelet agonist, are increased in IBD. The increased expression of PAR-1 in IBD patients already strongly suggested its role as a risk factor to induce platelet activation and consecutively thromboembolic disease. We here add another piece of evidence for the significant role of thrombin inducible platelet activation by showing an increased susceptibility for PAR mediated platelet activation in patients with active CD.

We choose whole blood impedance aggregometry for the evaluation of the aggregation response of platelets, because MEA is well standardized and data from one laboratory can easily be compared to those from another one. As minimal manipulation requirements by MEA are a major advantage, any artifacts are significantly reduced and all data can therefore be regarded highly specific. Previous reports indicated increased platelet aggregation in response to epinephrine, collagen and ADP in IBD patients. Our data complement these findings by showing an enhanced responsiveness specifically for PAR-inducible platelet aggregation.

However, as an overlap of results is seen, other pathways of platelet activation may be active as well in some patients. The role of PAR-1 inducible platelet activation is substantiated by the findings of significant stronger responsiveness to PAR-1 induced expression of P-selectin in patients with active disease. Activated platelets express P-selectin (CD62P) and bind rapidly to lymphocytes, monocytes, neutrophils and endothelium via the constitutively expressed P-selectin glycoprotein ligand-1 (PSGL-1) receptor. The interaction between P-selectin and its main receptor PSGL-1 leads to platelets adhering to leukocytes, which then become further activated and secrete inflammatory substances, like chemokines, including RANTES, platelet factor 4 and pro-angiogenic factors, including VEGF and PDGF. Our data indicate that PMA formation through PAR-1 and PAR-4 mediated platelet activation is high in active CD compared to controls. In concordance with the impedance aggregometry data, this marked responsiveness almost completely resolved in patients with CD in remission. This direct association of the clinically active disease, reflected by the HBI, with PAR mediated platelet activation and levels of PMA is of special importance, since PMA formation is associated with thromboembolic disease.

An enhanced platelet activation in IBD was shown by platelet activation markers such as P-selectin and measurements of $\beta$-thromboglobulin and by various reports showing an elevation of different platelet associated products, such as platelet factor 4 (PF4) and CD40 ligand.
Platelet activation in Crohn’s disease via PAR-1 and PAR-4

This platelet activation could be just from their active role in inflammation, without a specific engagement associated with thrombosis. However, the platelet response to specific stimuli results in diverse platelet responses, suggesting that platelets can discriminate between different stimuli. Thus, demonstrating increased levels of PAR-mediated platelets adhering to monocytes, a sensitive marker of thromboembolic disease and inflammation, bridges inflammation with thromboembolic disease in active CD.

We show that PAR-1 and PAR-4 inducible platelet aggregation and levels of PMA formation normalized in CD patients in remission. However, PAR-1 activated platelets of patients with CD express higher levels of surface P-selectin compared to controls, regardless the clinical disease activity. These findings are in line with previous reports showing activated platelets irrespective of the inflammatory status. One likely explanation for elevated P-selectin levels is that despite a Hb ≤ 4, subclinical inflammation persists, leading to high P-selectin expression even in a population with clinical remission. The question why we, in contrast to published data, could correlate platelet activation with disease activity remains to be elucidated. Possible explanations could be different used platelet tests and agonists as well as different used clinical markers to define disease activity. Andoh et. al used light transmission aggregometry which requires preparation of platelet rich plasma. This procedure, in contrast to whole blood MEA, leads to manipulation and possible in vitro activation of platelets thereby masking potential pathophysiological effects.

In contrast to PAR-1, PAR-4 mediated activation led to no significant differences in surface P-selectin expression with respect to the status of activation or in comparison to controls. The disparate observation of significant stronger response of PMA to PAR-4 mediated platelet activation in patients with active CD, but without such a stronger response of platelet surface P-selectin expression, may be due to other receptor–ligand associations for platelet–monocyte aggregate formation. We cannot exclude that we have underestimated other interactions between platelets and monocytes by focusing on the P-selectin-PSGL1-axis only.

Our study has some strengths and some limitations. We show for the first time, with the highly specific and well standardized whole blood impedance aggregometry (MEA), significantly higher platelet aggregation via thrombin receptors PAR-1 and PAR-4 in patients with active CD compared to patients in remission or controls. The choice of coeliac disease patients as controls could be discussed. However, these patients were symptom-free on gluten-free diet for at least 12 months and were anti-endomysial antibodies (EMA) and anti-tissue-transglutaminase antibodies — negative. Furthermore, in a previous study, we demonstrated that patients with coeliac disease are not at increased risk for venous thromboembolism. Usually patients with coeliac disease in long-term remission do not have any signs of significant inflammation in the gut or in the serum and are appropriate non-inflammatory controls. A limitation of this study is the lack of clinical outcome data with thromboembolic complications. No patient had any documented thromboembolic event in the medical history at the time of study entry. However, in this study we tried to complement current epidemiologic data on thromboembolic disease in CD with functional data of primary hemostasis. Further, our findings may not be generalized to UC as only platelets from CD patients were studied. It is however difficult to recruit patients with UC for studies on platelet activation, as most of these patients are on 5-aminosalicylic acid treatment, which may influence platelet function.

In conclusion, we demonstrate that PAR responsiveness is increased in CD and this effect can be correlated to clinical disease activity of CD. We may provide a biological foundation for the increased risk of thromboembolic disease in CD patients, that may translate into new diagnostic and therapeutic opportunities.

Conflict of interest

Each author disclose that he or she has no commercial associations (e.g., consultancies, stock ownership, equity interest, patent/licensing arrangements, etc.) that might pose a conflict of interest in connection with the submitted article.

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