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Original Article

Portal vein thrombosis and haematemesis in chronic liver disease. Are P-selectin and PSGL-1 clues?

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ABSTRACT

Background and study aims: Bleeding and thrombotic complications are common problems in patients with chronic liver disease (CLD). The aim of the present study was to evaluate the level of soluble P (sP)-selectin, and P-selectin glycoprotein ligand-1 (PSGL-1) (CD162) expression on neutrophils among patients with CLD and to clarify the role of their interaction, by measuring the platelet leucocyte aggregates, on the clinical outcome of the haemostatic balance in those patients. We also investigated the hypothesis that the balance between platelet activation and endothelial biological function is impaired.
Patients and methods: sP-selectin and thrombomodulin (TM) levels were measured by enzyme-linked immunosorbent assay (ELISA) and flowcytometric detection of CD162 was performed. Platelet–leucocyte aggregation (PLA) in whole blood was measured as positive for CD41a and CD45 in 66 CLD patients divided into the portal vein thrombosis group (PVT) ($n = 25$), the haematemesis group ($n = 21$) and the haemostatically stable group ($n = 20$).
Results: sP-selectin was significantly elevated in all patient groups. Decreased surface expression of CD162 on neutrophils was detected in all patients' groups. PLA was statistically significantly increased in the PVT group. TM was statistically significantly increased in the PVT, haematemesis and haemostatically stable groups.
Conclusion: PLA may play a role in the unique PVT outcome of the haemostatic balance in a group of patients whose credentials of hyperdynamic portal circulation predispose them to bleeding rather than thrombosis. Consequently, P-selectin targeted therapy may be used to prevent this complication.
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Introduction

The liver plays a central role in maintaining the critical balance between bleeding and thrombotic processes [1]. In liver cirrhosis, there is restrained adaptability of the haemostatic balance and depending on the triggering stimulus, the patient can develop thrombosis and/or haemorrhage [2]. Despite clear evidence of an increased tendency for bleeding in patients having cirrhosis, many circumstances also promote local and systemic hypercoagulable states. The consequences of hypercoagulability include the obvious morbidity and mortality of portal vein thrombosis, deep vein thrombosis and pulmonary embolism [3].

P-selectin (CD62P) is a 140 000-molecular-weight transmembrane glycoprotein, which was identified using monoclonal antibodies specific for thrombin-activated platelets. It is a member of the selectin family of adhesion molecules [4]. A soluble form of P-selectin, which might represent a proteolytic fragment or a soluble splice variant lacking the transmembrane domain, is found in serum and plasma [5]. P-selectin has been shown to be involved in thrombogenesis as well as bleeding disorders and may represent a possible link between inflammation and thrombosis [6].

P-selectin glycoprotein ligand-1 (PSGL-1, CD162) is a mucin-like cell adhesion molecule expressed on leucocyte plasma membranes and is involved in platelet–leucocyte and endothelium–leucocyte interactions [7]. It is a widely distributed adhesion molecule that plays a critical role in regulating lymphocyte homing and leucocyte trafficking during inflammation [8].

Recent published data have confirmed that platelets are not only members of the coagulation system but they also play a pivotal role in promoting immune responses and initiating inflammation. The pro-inflammatory functions of platelets are based on two major events. The first well-known function is the release of stored biological active mediators from their granules to promote inflammation. Other striking findings were receptor-mediated cross-talk

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between platelets and different immunocytes, including platelet–neutrophil aggregation [9].

Activation of platelets enhance platelet P-selectin expression, which may bind to leucocytes and form platelet–leucocyte aggregation (PLA), mainly through coherence of platelet P-selectin with leucocyte P-selectin glycoprotein ligand-1 (PSGL-1), which is constitutively expressed on the leucocyte surface. Platelet P-selectin expression and PLA formation have been reported to be potential markers of inflammatory diseases such as sepsis and thrombosis [10].

Thrombomodulin (TM) is a marker of endothelial function. TM is expressed mainly on the surface of vascular endothelial cells. It suppresses blood coagulation, and is a key component of the protein C anticoagulant pathway. TM converts thrombin from a procoagulant to an anticoagulant protease by increasing the rate of protein C activation [11].

The aim of the present study was to evaluate the level of sP-selectin and PSGL-1 (CD162) expression on neutrophils among patients with chronic liver disease (CLD) and to clarify the role of their interaction, by measuring the platelet–leucocyte aggregates, on the clinical outcome of the haemostatic balance in those patients. We also investigated the hypothesis that the balance between platelet activation and endothelial biological function is impaired.

Patients and methods

This study was approved by the ethical committee of Theodor Bilharz Research Institute (TBRI). A written consent was taken from all patients and control subjects according to Helsinki guidelines. This study included 66 patients with CLD, admitted to Tropical Medicine Department at TBRI. Clinical, laboratory and radiological evaluation of patients were done and, consequently, patients were categorised as Child C group. Hepatocellular carcinoma was excluded in all patients by the absence of focal lesion by abdominal spiral computed tomography and by normal levels of alpha fetoprotein. The aetiology of CLD was hepatitis C (HCV) and B (HBV) viruses as diagnosed by the presence of viral markers by enzyme-linked immunosorbent assay (ELISA) and confirmed by polymerase chain reaction (PCR).

Patients were classified into three groups: portal vein thrombosis group ($n = 25$), the haematemesis group ($n = 21$) and the haemostatically stable group ($n = 20$).

The first group included patients with CLD who were proved to have portal vein thrombosis (PVT) by abdominal ultrasonograph (US) and duplex colour Doppler sonography. The second group, the haematemesis group, which included 21 patients with CLD, with a history of haematemesis due to ruptured oesophageal varices during the last 24 h. Samples of these patients were collected before administration of any parenteral therapy. The third group comprised patients who neither had a history of haematemesis nor proved to have PVT. These patients were under conservative treatment, including liver support, beta blockers, vitamin K and albumin. All patients and control subjects were subjected to full clinical evaluation, liver function tests, excretory (bilirubin) and synthetic (prothrombin time, concentration and albumin), complete blood count (CBC), abdominal ultrasonograph (US), duplex colour Doppler sonography and upper gastrointestinal endoscopy. In addition, 20 age- and gender-matched healthy individuals were enrolled as a control group. All participants of the control group had normal blood counts and liver function tests.

Specimen collection and handling

Blood samples were collected from an antecubital vein of patients and control subjects under aseptic conditions using an 18-gauge needle without using a tourniquet. About 6 ml of blood was withdrawn from each patient. The sample was divided into the following three portions: 2 ml of blood was delivered into an ethylene diamine tetraacetic acid (EDTA) vacutainer to perform the routine haemogram and platelet count, 2-ml samples were anticoagulated with 1:9 volume of 3.8% sodium citrate solution. Citrate anticoagulated blood was divided into two tubes, the first of which was centrifuged at 3000 rpm and the plasma was immediately used for coagulation profile assay (prothrombin time (PT), partial prothromboplastin time (PTT)) and TM. The second part was processed for flowcytometer analyses. Two millilitres of blood were delivered in a gel-containing vacutainer and allowed to clot at room temperature. The serum was separated by centrifugation at 2000 rpm for 10 min and used for assessing liver function tests, hepatitis B and C markers and sP-selectin assay.

Procedure of the technique for immunophenotyping [12]

One-hundred microlitres of the citrate anticoagulated blood was dispensed into each of three sterile Wassermann tubes: The first tube was the control for the measurement of non-specific binding of mouse monoclonal antibody of isotype IgG1 to human tissues and to adjust the auto-fluorescence region. In the second tube, 10 μl of anti-CD162 monoclonal antibody (phycoerythrin (PE) Mouse Anti-Human CD162 supplied from DAKO) was added and mixed with blood. The third tube was used to determine PLAs. Blood samples were mixed with saturated concentrations of anti-CD45-fluorescein isothiocyanate (FITC) monoclonal antibody and anti-CD41a-PE monoclonal antibody. PLA in whole blood was measured as positive for CD41a and CD45. Leucocytes were identified by their anti-CD45-FITC fluorescence and differentiated into subgroups based on cell size and granularity in the forward and side scatter. The three tubes were incubated for 15 min at 4 °C in the dark. As much as 2 ml of diluted lysing reagent (EasyLyse™) was added to each tube; then, the sample was incubated with the lysing reagent for 10–15 min at room temperature according to the 'Lyse no wash protocol' [13]. The sample was run on the flowcytometer within 45 min. The flowcytometer Epics R Elite Coulter system was used for flowcytometric analysis. A total of 5000 gated cells were counted where the monoclonal tagged with PE was analysed on PMT2 (green) and the monoclonal tagged with FITC was analysed on PMT1 (red). The results were declared as percentage of CD162 cells inside the gated population of neutrophils. Results of PLA were expressed as mean fluorescence intensity (MFI).

Soluble P-selectin and TM were assayed by enzyme-linked immunosorbent assay (ELISA) according to manufacturer guidelines using R&D Systems, Human sP-Selectin ELISA kit and Euroclone Life Science Division, Human sCD141 ELISA kit, respectively [14,15].

Statistical methodology

Statistical Package for Social Science (SPSS) version 9.0 and Grafpade program were used for analysis of data. Data were summarised as mean, SD and percentage. Non-parametric (Mann–Whitney *U*) test was used for analysis of quantitative data, as data were not symmetrically distributed. While the chi-square test was used for analysis of qualitative data, the Kruskal–Wallis *H* test was done for analysis of more than two quantitative data. *P*-value less than 0.05 was considered significant. Correlation analysis was done to assess the relation between different parameters. Regression analysis was done to determine the independent factors of PVT.

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