Research Article

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Platelet production and destruction in liver cirrhosis

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Background & Aims: Thrombocytopenia is common in liver cirrhosis (LC) but the mechanisms are not fully understood. The purpose of our work was to evaluate platelet kinetics in LC with different etiologies by examining platelet production and destruction.

Methods: Ninety-one consecutive LC patients (36 HCV, 49 alcoholics, 15 HBV) were enrolled. As controls, 25 subjects with idiopathic thrombocytopenic purpura, 10 subjects with aplastic anemia, and 40 healthy blood donors were studied. Plasma thrombopoietin (TPO) was measured by ELISA. Reticulated platelets (RP) were determined using the Thiazole Orange method. Plasma glycocalicin (GC) was measured using monoclonal antibodies. Platelet associated and serum antiplatelet antibodies were detected by flow cytometry. B-cell monoclonality in PBMC was assessed by immunoglobulin fingerprinting.

Results: Serum TPO was significantly lower in LC (29.9 ± 18.1 pg/ml) compared to controls (82.3 ± 47.6 pg/ml). The GC levels were higher in LC (any etiology) than in healthy cases. Conversely, the absolute levels of RP were lower in LC (any etiology) than in healthy controls. The platelet-associated and serum anti-platelet antibodies were higher in HCV+ LC compared to healthy subjects (p <0.0064), alcoholic LC (p <0.018), and HBV+ LC (p <0.0001). B-cell monoclonality was found in 27% of the HCV + LC, while it was not found in HBV+ or alcoholic LC.

Conclusions: Patients with LC present decreased plasma TPO, accelerated platelet turnover, and reduced platelet production. This indicates that LC thrombocytopenia is a multifactorial condition involving both increased platelet clearance and impaired thrombopoiesis.

Keywords: Liver cirrhosis; Glycocalicin; Thrombopoietin; HCV; HBV; Aplastic anemia; Idiopathic thrombocytopenic purpura.

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Abbreviations: LC, liver cirrhosis; HCV, hepatitis C virus; HBV, hepatitis B virus; TPO, thrombopoietin; RP, reticulated platelets; GC, glycocalicin; ITP, idiopathic thrombocytopenic purpura; AA, aplastic anemia; PBMC, peripheral blood mononuclear cells; TBS, Tris-buffered saline; PRP, platelet-rich plasma; BSA, bovin serum albumin; ACD, acid Na-citrate dextrose.

Introduction

Thrombocytopenia is a frequent feature in patients with liver cirrhosis (LC). It has traditionally been attributed to the sequestration of platelets by the spleen [1–3], a situation known as "hypersplenism" secondary to portal hypertension. Nevertheless, no clear correlation between the portal pressure and the platelet count has ever been observed. Furthermore, in some cirrhotic patients thrombocytopenia may persist even after splenectomy or after portal decompression [4–9]. Since the return to a normal platelet count has been observed following liver transplantation [10], it is likely that other mechanisms apart from hypersplenism, such as reduced thrombopoietin (the cytokine which regulates megakaryocyte maturation and platelet production) release by the liver [11–14] or bone marrow suppression [15,16], are involved in the thrombocytopenia of cirrhotic patients.

To increase the complexity of this problem, the etiology of the liver disease should be taken into account since HBV, HCV, and ethanol (the most common causes of liver cirrhosis) all induce liver damage via different mechanisms; therefore, even if the final outcome is the same (cirrhosis), the biochemical and immunological abnormalities are quite different, especially in HCV-infected patients. In addition, the possibility for studying platelet turnover "*in vivo*" is limited. In this study, we assessed reticulated platelet counts and glycocalicin levels as measures of platelet production and destruction, respectively. We also quantified levels of serum thrombopoietin and anti-platelet antibodies in groups of patients affected by cirrhosis of different etiologies (HBV-related, HCV-related or alcohol induced-LC) and in two control groups of patients affected by hematological diseases known to affect the platelet count, such as idiopathic thrombocytopenic purpura (ITP) and aplastic anemia (AA), as well as in healthy control subjects. In a fraction of the patients, we assessed for the presence of an expanded monoclonal B-cell population in the peripheral blood mononuclear cells (PBMC) and for immunoglobulin gene usage via isotype-specific immunoglobulin fingerprinting.

Patients and methods

Hepatological patients

Ninety-one consecutive patients with liver cirrhosis and platelet counts less than 100×10^9/L were enrolled in the study. Of these patients, 36 had a HCV-related liver disease, 40 were alcoholics, and 15 had a HBV-related liver disease. None of the patients had previously undergone interferon therapy or were taking drugs

known to interfere with bone marrow function. The diagnosis of cirrhosis was based on the grounds of either liver biopsy (58 cases, 64%) or on findings resulting from a physical examination of the patient, results of laboratory tests, imaging studies, and the presence of portal hypertension (splenomegaly, ascites, and esophageal varices). The liver biopsies were placed in buffered formalin and stained with haematoxylin and eosin or with Gomori methenamine silver for reticulum staining. In HCV and HBV patients, the disease activity and fibrosis were assessed according to the METAVIR scoring system [17]. Patients carrying anti-nuclear, anti-mitochondrial, or anti-smooth muscle antibodies were discarded from the study.

The study protocol was approved by the ethical committee and conforms to the ethical guidelines of the 1975 Declaration of Helsinki. All patients gave written informed consent for participation in this medical research.

Hematological patients

Twenty-five cases of idiopathic thrombocytopenic purpura (ITP) were enrolled in the study. Each subject presented the active phase of the disease but had not yet initiated immunosuppressive treatment. This disease is characterized by a high platelet turnover and the presence of anti-platelet antibodies. The diagnosis of ITP was based on commonly adopted criteria [18] involving: the patient's medical history, physical examination report, complete blood cell count, and cytomorphologic examination of the peripheral-blood smear in which no alterations of erythrocytic and leukocytic series should be present. To confirm the diagnosis, bone marrow aspiration was performed on all patients and only those with the presence of a normal or increased number of megakaryocytes without pathologic alterations of the erythroblastic, granuloblastic, or lymphocytic series were included in the study. Moreover, we had the opportunity to study 10 patients affected by severe aplastic anemia, a disease exhibiting a very low platelet count due to a reduced platelet production. The definition of ''severe'' aplastic anemia was determined according to currently used criteria [19]: the disease was considered severe if at least two of the following were noted: a neutrophil count less than 0.5×10^9/L, a platelet count less than 20×10^9/L, and a reticulocyte count less than 60×10^9/L with hypocellular (<20%) bone marrow.

Controls

Forty volunteer blood donors, serologically negative for HIV, HBV, and HCV, with normal platelet counts were used as normal healthy control subjects. Informed consent was obtained prior to all blood donations.

Methods

In this observational study, we had the opportunity to apply the same up-to-date methods for studying platelet turnover to different groups of patients. The anti-HCV antibodies were detected by a third-generation enzyme immunoassay (Ortho HCV SAVe 3.0, Raritan, NJ). Positivity for anti-HCV antibodies was confirmed by strip immunoblot assay (RIBA HCV 3.0, Chiron Corp., Emeryville, CA). In anti-HCV-positive patients, the serum HCV-RNA was assessed by means of nested reverse transcription polymerase chain reaction. Commercially available enzyme-linked immunoassays were used to measure HBeAg, anti-HBe, HbsAg, and anti-HBs (Abbott Laboratories, USA). The Serum HBV-DNA was measured using the TaqMan polymerase chain reaction assay (COBAS TaqMan, Roche Molecular System, lowest limit of detection: 70 copies/ml).

The maximum longitudinal diameter of the spleen was estimated by means of an ultrasound scan by an experienced operator. Ultrasonography was carried out using an AU 570 Asynchronous (Esaote Biomedica, Genoa, Italy).

Reticulated platelets

Peripheral blood was collected using ACD-A (at the ratio 8.5/1.5 v/v) and centrifuged at 100*g* for 10 min at 22 °C to obtain platelet-rich plasma (PRP). 10 μl PRP from normal subjects and 20–50 μl from all other patients were added to 1 mL of Thiazole Orange diluted 1:4 in Tris-buffered saline, pH 7.4, 10 mM EDTA, 0.1% bovine serum albumin (TBS/EDTA/BSA), and incubated for 60 min at 22 °C in the dark. PRP incubated with TBS/EDTA/BSA without Thiazole Orange was used as a blank for each sample. Fluorescence was analysed using an Epics-Elite flow cytometer (Coulter, USA). Logarithmic amplification was used for both light scatter and fluorescence signals. For each sample, fluorescence signals were collected from 10,000 cells and a frequency histogram created. Three normal controls were run in each assay; a threshold was positioned giving 1% of fluorescent platelets in healthy subject control samples. The same threshold was used for the analysis of all samples. The percentage of reticulated platelets was obtained by subtracting the percentage of reticulated platelets of unstained preparations from that of stained samples. A normal reference range (5.9 ± 2.25) was obtained using blood samples from 60 healthy normal subjects.

Glycocalicin assay

Blood was collected in EDTA at a 50 mM final concentration. The samples were processed within 2 h following blood collection since preliminary tests showed that no change in glycocalicin values could be observed within this time period. The plasma was centrifuged at 12,000*g* for 5 min and the supernatant was stored at −70 °C. The monoclonal antibodies LJ-P3 and LJ-P19 were kindly given by Z.M. Ruggeri (Scripps Clinics, La Jolla, CA, USA). LJ-P3 and LJ-P19 monoclonal antibodies interact with epitopes only present in the native glycoproteins located within residues 1-290. The epitopes for the two monoclonal antibodies are distinct since no cross-reactivity was observed. To determine the specificity of each monoclonal antibody, we measured glycocalicin (GC) concentration in pooled control plasma from normal subjects by co-incubating the plasma with various concentrations of mouse IgG and LJ-P19. There was no decrease in the concentration of GC following co-incubation of the plasma at different dilutions (1:500–1:25,000) of mouse IgG at room temperature for 1 h. By contrast, the GC concentration was decreased in plasma co-incubated in concentrations of nonbiotinylated LJ-P19 ranging between 3.3 and 0.066 μg/ml. GC was purified as previously described by Vicente et al. [20]. GC concentration was determined by the Micro BCA-method (Pierce, Rockford, USA) using albumin as a standard. The GC value could be underestimated by approximately 30–40% in the protein assay because of the high glycosylation of GC. Five to eight fresh random platelet concentrates, to which a 15% acid Na-citrate dextrose (ACD) solution was added, were centrifuged at 800*g* for 20 min at room temperature. The platelet pellet was then washed twice with TBS, pH 7.4, containing 2 mM EDTA and 15% ACD, resuspended in TBS containing 2 mM $CaCl_2$ and 0.1 mM phenyl-methyl-sulphonyl-fluoride and sonicated on ice. The suspension was then stirred (2 h at RT and 18 h at 4 °C) and centrifuged at 23,000*g* for 30 min. The supernatant was applied to an affinity column of wheat germ agglutinin-Sepharose 6 MB (Pharmacia, Uppala, Sweden), and the bound proteins were eluted with TBS containing 1 mM EDTA, 0.1 mM PMSF, 3 mM NaN_3, and 100 mM *N*-acetyl-glucosamine. The eluate was then immunopurified with an LJ-P3 Sepharose column (Pharmacia, Uppala, Sweden). GC was eluted with 50 mM diethylamine, 1 mM EDTA, and 0.1 mM PMSF. Glycine 0.55 M was added and the eluted protein was dialysed for 18 h against TBS and concentrated with Centiprep 100. Purified GC was stored at −80 °C. GC was assayed in plasma by ELISA following the methods of Kunishima et al. [21] and Beer et al. [22], with some modifications. Briefly, a flat-bottomed microtitre plate (Costar, Cambridge, USA) was coated with the monoclonal antibody LJ-P3 (1.0 μg/ml) overnight at 4 °C, washed 3 times with PBS containing 0.05% Tween (PBS-T), blocked with PBS containing 2% BSA and washed again with PBS-T. The standard curve was prepared using immunopurified GC ranging from 0.05 to 1 μg/ml in PBS-T. The plasma samples were then diluted 4-fold and 8-fold in PBS-T and 100 μl of each added to individual wells. After incubation for 60 min at RT, 100 μl of the biotin-labelled LJ-P19 (1.5 μg/ml in PBS-T) were added and incubated for a further 60 min. After three washes, each well was filled with 50 μl of the streptavidin-HRPO (Caltag, South San Francisco, CA, USA), diluted 1000-fold in PBS-T, and incubated for 60 min at RT. After three washes, 100 μl of the *o*-phenylenediamine dihydrochloride reaction was stopped by adding 50 μl of $2NH_2SO_4$ and the absorbance was measured at 492 nm with a microtitre plate reader. The results were plotted and expressed as GC concentration in μg/ml and as GC indices. The GC index represents the standardization of the GC value for a platelet count of 250×10^9/L and is calculated as follows: GC index = GC μg/ml × (250×10^9/L/patient's platelets).

Thrombopoietin

Serum thrombopoietin (TPO) levels (pg/ml) were measured by means of ELISA, (Quantikine. R&D Systems Europe Ltd., Oxon, UK) according to the manufacturer's instructions. The lower limit of detection of the kit is 15 pg/ml. Sera were collected in a serum-separator tube and allowed to clot for approximately 45–60 min; they were then centrifuged, chilled rapidly, and stored at −20 °C until TPO determination. In our laboratory the intra-assay coefficient of variation was 8.7%.

Detection of antiplatelet antibodies by flow cytometry

The direct labeling procedures for platelet-associated Ig, and the indirect labeling procedures for serum circulating Ig, were performed according standard procedures.