Platelet production and destruction in liver cirrhosis

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Abstract

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.

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

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 x 10⁹/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 Comori 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 erythrocytes or leukocytes were evidenced by microscopy. To confirm the diagnosis of 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 assayed by means of nested reverse transcription polymerase chain reaction. Commercially available enzyme-linked immunosorbant assays were used to measure HBeAg, anti-HBc, HbsAg, and anti-HBs (Abbott Laboratories, USA). The serum HBV-DNA was measured using enzyme-linked immunoassays were used to measure HBeAg, anti-HBe, HbsAg, and anti-HBs (Abbott Laboratories, USA). The Serum HBV-DNA was measured using enzyme-linked immunoassays were used to measure HBeAg, anti-HBe, HbsAg, and anti-HBs (Abbott Laboratories, USA). 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Using an EPICS-Elite flow cytometer (Coulter) equipped with a 488 nm argon-laser, data were acquired as 5000 ungated events and a flow rate of 150–300 events was maintained. Platelets were gated using forward- and side-angle light scatter and log amplification with the gains adjusted to include the whole platelet population. Fluorescent signals were then obtained with platelets sensitized to surface IgG (direct method), patients, and control samples (indirect method).

B-cell monoclonality

Total cellular RNA was isolated from PBMC using the procedure described by Chomczynski and Sacchi [23]. PBMC was obtained by fractionation of whole blood (or bone marrow) on the Ficoll/Hypaque gradient. One μg of mRNA was reverse transcribed (RT) using random examers. The entire RT product was next ligated into a Sma site of a pUC18 plasmid, and used to transform the Escherichia coli strain DH5a. After expansion, the clones were randomly picked and double-stranded DNA templates were sequenced using the T7 sequencing kit. The procedure was performed in 30 HCV and 10 HBV cirrhosis patients, and 10 patients with alcoholic cirrhosis.

Statistics

Statistical analysis was carried out using the Chi-square-test to compare proportions and the Mann–Whitney U-test to compare means. Spearman’s rank correlation tests were used to look for correlations between the various parameters. Data are shown as the mean ± SD. A p value of 0.05 or less was considered statistically significant.

Results

The main characteristics of the 80 patients with liver cirrhosis are given in Table 1. As shown, the three groups of patients had similar clinical, hematological, and biochemical characteristics. The clinical data setting of the hematological patients is indicated in Table 2.

Reticulated platelets

In the patients affected by alcoholic cirrhosis, the mean percentage of reticulated platelets was very similar to that found in normal healthy subjects (5.8 ± 3.2% vs. 5.9 ± 2.2% p: NS). Given the reduced platelet count (76.826 ± 26.020 × 10^9/L) the absolute levels of reticulated platelets were significantly higher in normal subjects compared to patients with alcoholic cirrhosis (4.233 ± 2.367 × 10^9/L vs. 14.666 ± 5.999 × 10^9/L p < 0.0000000012). Similar results have been found in HBV+ liver cirrhosis (percentage of reticulated platelets 5.5 ± 4.1% with absolute levels of 4.996 ± 3.143 × 10^9/L). In HCV+ liver cirrhosis, despite a similar mean platelet count (67.811 ± 19.181 × 10^9/L p: NS), the percentage of reticulated platelets was significantly higher (11.3 ± 4.7%) than in patients with alcoholic cirrhosis (5.8 ± 3.2% p < 0.043) and HBV+ cirrhosis (5.5 ± 4.1% p < 0.05). Consequently, the absolute counts of reticulated platelets were higher in HCV patients (6.629 ± 7.408 × 10^9/L) than in either patients of alcoholic cirrhosis (4.233 ± 2.367 × 10^9/L) or HBV (4.996 ± 3.143 × 10^9/L); given the high standard deviation of the HCV patients, the percentage (with HBV of alcoholic cirrhosis) does not reach statistical significance (p = 0.068). In the HCV patients, two groups can be identified, the first one (27 cases) with low reticulated platelet count (4.3 ± 1.7 i.e. 3.171 ± 1.572 × 10^9/L) and second one (nine cases) with normal-high reticulated platelet count (31.6 ± 17.6% i.e. 16.640 ± 9.377 × 10^9/L p < 0.0000000027).

The patients affected by ITP showed very high levels of reticulated platelets (25.7 ± 11.2%), while very low levels were found in the patients with aplastic anemia (1.0 ± 0.9%). The results obtained from these hematological cases are in line with the pathophysiology of the underlying diseases: increased platelet production for accelerated destruction in the former and very low production in the latter.

Glycocalcin

In the normal healthy subjects, given the slow rate of platelet turnover, the glycocalcin index was within normal limits (0.9 ± 0.2), while in the patients with liver cirrhosis, independent of etiology, the glycocalcin index was high: 1.96 ± 1.40 in HCV+ cirrhosis (p < 0.0005 vs. healthy controls), 1.79 ± 1.51 in the alcoholics (p < 0.006 vs. healthy controls) and 1.71 ± 1.69 in HBV+ cirrhosis (p < 0.006 vs. healthy controls). In the HCV patients, even for the glycocalcin index, two groups can be identified: the first one (27 cases) with a value comparable to the alcohols and HBV+ cirrhosis (1.76 ± 1.48) and a second one (nine cases) with high glycocalcin index (2.46 ± 0.84; p < 0.02). The mean glycocalcin level and index were significantly greater in patients affected by ITP (12.9 ± 4.4, p < 0.000002 vs. healthy controls). In the patients with aplastic anemia, the glycocalcin index was normal (0.8 ± 0.1), in accordance with the normal platelet turnover.

Thrombopoietin

The serum thrombopoietin levels were significantly (p < 0.0003) lower in patients with cirrhosis (29.9 ± 18.1 ng/ml) compared to healthy controls (94.7 ± 35.9 ng/ml). No differences were found on the basis of the etiology of the liver cirrhosis (29.2 ± 16.2 in the alcohols, 30.6 ± 22.0 in HCV+ and 31.1 ± 19.9 in HBV+). On the contrary, levels above the normal limits were found in the patients with aplastic anemia (507.7 ± 86.1 pg/ml) and in patients affected by ITP (155 ± 76 pg/ml). The thrombopoietin serum levels were inversely correlated to platelet counts in ITP patients (r: -0.87), while this correlation was not found in patients with liver cirrhosis. Moreover, no difference was observed in TPO serum levels between patients with or without splenomegaly, and no correlation was observed between TPO serum levels and either spleen longitudinal diameter or surface area, or liver histological inflammatory activity (in both HCV and HBV subjects) (data not shown).

Spleen size

Seventy percent of the cirrhosis patients presented splenomegaly (i.e. a spleen longitudinal diameter over 12 cm). The level of thrombocytopenia was not correlated to either spleen size (considering both spleen longitudinal diameter and spleen surface area) or liver histological necro-inflammatory activity (in both HCV and HBV patients). The spleen size was normal in all patients with ITP and aplastic anemia.

Anti-platelet antibodies

Normal healthy subjects were not found to express any detectable levels of anti-platelet antibodies. As expected, most patients (85%) affected by ITP presented platelet-associated antibodies, and a large fraction of them (65%) also expressed serum circulating anti-platelet antibodies. None of the patients with aplastic anemia expressed anti-platelet antibodies. The frequencies of
platelet-associated antibodies in patients with liver cirrhosis (all etiologies combines) and patients with ITP were significantly higher compared to healthy controls (p < 0.005 and 0.0001, respectively). Among the patients with HCV-related cirrhosis, platelet-associated antibodies were found in seven cases (19.4%) and serum circulating anti-platelet antibodies in nine cases (25.0%). However, five patients had both platelet-associated and circulating anti-platelet antibodies, while four patients only expressed serum circulating antibodies and two cases expressed platelet-associated antibodies only. In alcoholic cirrhosis, only one case was found to have platelet-associated antibodies (2.5%) and two cases had circulating anti-platelet antibodies (5.0%); even in HBV-related cirrhosis, only one patient showed serum anti-platelet antibodies (6.6%), while no cases presenting platelet-associated antibodies were found. Statistical analysis showed that the prevalence of platelet-associated antibodies was significantly higher in HCV+ cirrhosis compared to healthy subjects (p < 0.0064), alcoholic cirrhosis (p < 0.018), and HBV+ cirrhosis (p < 0.0001). Even the prevalence of circulating anti-platelet antibodies was significantly higher in HCV+ cirrhosis compared to normal controls (p < 0.0021), alcoholic cirrhosis (p < 0.0152), and HBV+ cirrhosis (p < 0.006).

B-cell monoclonality

B-cell monoclonality was found in eight (27%) of the HCV-positive patients, whereas no monoclonality was found in HBV (p < 0.004) or alcoholic patients (p < 0.003). The five HCV+ patients with both serum and platelet-associated antibodies and one out of the four patients with serum circulating antibodies showed B-cell monoclonality, while the remaining two HCV+ cases with B-cell monoclonality also suffered from other autoimmune diseases i.e. Coombs-positive hemolytic anemia and autoimmune thyroiditis. In all these cases, a skewed immunoglobulin usage was found; in fact, these cases presented a preferential usage of the monoclonal 51p1 gene. The sequence of the variable regions (Complementary Determinant Regions) of the immunoglobulins showed a region of a different length with a different nucleotide and amino acid sequence in each case.

Discussion

The possibility to compare hematological and hepatological patients allowed us to better understand the complex mechanisms of platelet turnover. Thrombocytopenia is the main hematological disorder observed in liver cirrhosis, and this condition has been traditionally attributed to hypersplenism [24], a condition accompanied by an increase in the sequestration and an accelerated destruction of platelets. However, improvements in thrombocytopenia have not been achieved through the use of portal decompression, including portosystemic shunting, or even after splenectomy. Alternatively, the insufficient production of thrombopoietin has also been proposed to be involved in the pathogenesis of thrombocytopenia in patients with LC. Indeed, thrombopoietin, the principal stimulating factor of megakaryothrombopoiesis, is produced by the liver and its production is impaired in advanced liver failure [25–28]. The circulating level of thrombopoietin is known to be regulated by a “sponge effect”; this refers to the fact that free levels are controlled through its
binding to the thrombopoietin receptor that is mainly expressed on bone marrow megakaryocytes and circulating platelets, while thrombopoietin continues to be produced at constant rate by the liver [29]. Thus, the circulating levels of thrombopoietin depend upon total receptor numbers (at least in subjects with normal liver function). Indeed, it has been observed that following liver transplantation, serum thrombopoietin concentrations return to normal levels in conjunction with an increase in platelet count [30–32]. However, several studies have showed that some patients with liver cirrhosis have normal serum thrombopoietin levels despite low platelet counts. Our data support the hypothesis of a role of low thrombopoietin levels in cirrhosis thrombocytopenia, since its serum levels were found to be below normal levels in most cases. On the contrary, in a recent paper, Kajihara et al. [33] did not find any significant differences in the thrombopoietin levels between patients with liver cirrhosis and controls. These different findings could be explained by the different cut-off limit used for the platelets (100 x 10^9/L in our cases and 150 x 10^9/L in the others) and by the different prevalence of hepatocellular carcinoma (1% in our cases vs. 63% in those of Kajihara et al.). There are also some reports indicating that thrombopoietin receptors are expressed in fetal liver and in some hepatocellular carcinoma (HCC) cell lines [34]; however, other authors have observed increased platelet levels in HCC [35], thus such a high prevalence of HCC should be avoided in physiological studies.

Some rather old observations [36–39] exist indicating increased levels of immunoglobulin G (IgG) bound to platelets in patients with chronic liver disease that suggest the presence of autoantibodies reactive to platelets. Immunoglobulin bound to platelets is also found in idiopathic thrombocytopenic purpura (ITP) [40,1], a typical autoimmune disease characterized by increased platelet destruction mediated by autoantibodies against several platelet surface antigens, including GPIIb-IIIa (being the most common) and GPIb-IX [42,43]. ITP is characterized by accelerated platelet turnover (high glycoprotein levels) and thus, in turn, by an increased platelet production (high reticulated platelet count). Glycocalcin is a fragment of the platelet membrane glycoprotein Ib, and the glycocalcin index (GC index: the plasma GC level corrected for the platelet count) is considered to be a parameter of peripheral platelet turnover [44]. Since it was recently found that most patients with either liver cirrhosis (99% of cases) or ITP (94% of cases) present circulating anti-GPIIb-IIIa antibody-producing B cells [45], the immune-mediated destruction of platelets could be considered as the main factor determining thrombocytopenia in cirrhosis. Accordingly, we also found that mean GC levels and GC indices were abnormally high in patients with liver cirrhosis, suggesting an accelerated platelet turnover, though not at the same levels as in ITP. In patients subgrouped according to the etiology of the cirrhosis, the glycocalcin index was significantly higher in the patients affected by HCV compared to patients with other aetiologies. In line with these data, the prevalence of anti-platelet antibodies was significantly higher in HCV+ cirrhosis than in HBV+ or alcoholic cirrhosis. This difference is not surprising as it has been known for many years that chronic HCV infection determines several immunological (thyroid dysfunctions, Sjogren disease, and lichen ruber planus) [46,47] and hematological disorders (monoclonal gammopathies of undetermined significance, non-Hodgkin's lymphoma, Waldenström macroglobulinemia) [48–50]; although the strongest association has been found with mixed cryoglobulinemia [51,52]. The interaction of HCV envelope proteins and CD81, a tetraspanin mainly expressed on B-lymphocytes, seems to be the major mechanism of naive B-cell activation [53]. Note, the absence of any difference between HCV-positive and negative cases in auto-antibody production in Japanese patients can be explained by the ethnical difference in the immunological response. In fact, while HCV infection determines B–cell monoclonality in about 25% of chronically infected Caucasian patients, this does not occur in Japanese subjects [54]. Though the presence of detectable anti-platelet antibodies does not necessarily imply an accelerated platelet destruction, in this series of cases the subjects expressing anti-platelet antibodies (16 cases: seven with detectable platelet-associated antibodies and nine cases with serum circulating anti-platelet antibodies) showed a very high GC index (2.51 ± 1.26, p <0.06 vs. HCV+ cirrhosis without anti-platelet antibodies) and a very high level of reticulated platelets (11,962 ± 10,258/mm, p <0.05 vs. HCV+ cirrhosis without anti-platelet antibodies). On the basis of these results, as previously indicated in the “Results” section, in the HCV+ cirrhosis, two groups can be identified: in the first one (75% of the cases) the platelet production and the platelet turnover overlaps HBV+ and alcoholic cirrhosis, while the second one (25% of the cases) is characterized by high reticulated platelet, high GC index, high incidence of anti-platelet antibodies, and high incidence of B-cell monoclonality. Given the comparable severity of liver disease, the serum thrombopoietin levels were low in both groups (31.1 ± 16.7 vs. 30.5 ± 23.8 pg/ml; p: NS), this determines a platelet count significantly (p <0.02) lower in the cases with high platelet turnover (53,889 ± 11,241 x 10^12/L) than in the cases with standard platelet turnover (72,631 ± 19,131 x 10^12/L).

A quantitative analysis of megakaryocyte concentrations in bone marrow was not performed in this study. The identification of megakaryocytic cells based on morphological studies at the light microscopy is difficult and results are therefore often unreliable. In fact, while the identification of the mature megakaryocytes at different stages of development (given their large size, complex nuclear appearance, and typical cytoplasmic staining) is easy, even using light microscopy, the identification of immature megakaryocytic cells, which are small and do not have the classical and obvious features of megakaryocytes, generally requires ultrastructural studies and the use of electron microscopy for quantification. Alternatively, dual-color immunofluorescence staining and flow cytometry seems to be a reliable method to quantify bone marrow megakaryocytes at any stage of differentiation [55]. On the basis of these considerations, megakaryocyte quantification is even more difficult when the platelet turnover is elevated and, as a consequence, the immature fraction of small megakaryocytes is likely to be larger than in normal subjects. Therefore, the analysis of marrow megakaryocytes by the conventional light microscopy method is not reliable and the comparison of marrow megakaryocyte density in liver cirrhosis to that in ITP is unreliable.

In conclusion, patients with liver cirrhosis presented a normal or decreased plasma TPO, an accelerated platelet turnover (on the basis of a high glycocalcin index), and low or normal platelet production (on the basis of the absolute reticulated platelet count). Taken together, these findings indicate that cirrhotic thrombocytopenia is a multifactorial condition, involving both increased platelet clearance in the periphery and impaired thrombopoiesis. This is similar to what may happen in ITP since recent studies have shown that anti-GPIIb/IIIa autoantibodies were able to suppress
expression of megakaryogenesis [56,57]. This could explain the unusual finding of low levels of reticulated platelets and normal glycolytic indices in “classical” ITP subjects. In these cases, the growth factor-mediated stimulation of megakaryopoiesis might be expected to increase the platelet count. Indeed, initial clinical trials of the investigational thrombopoietic agent Eltrombopag (SB-497115, GlaxoSmitkline) a small-molecule, non-peptide that acts as a thrombopoietin-receptor agonist inducing proliferation and differentiation of megakaryocytes, were found to show promising responses in adults with chronic ITP refractory to other treatments [58,59]. In liver cirrhosis, this agent was found to increase the platelet count in HCV-related cirrhosis and to facilitate antiviral treatment with fairly good results [60]. This treatment might also be effective in advanced liver failure exhibiting thrombocytopenia and bleeding.

Finally, at least in Caucasians, a fraction of HCV-related liver cirrhosis is characterized by an increased prevalence of autoimmune phenomena, including detectable levels of anti-platelet antibodies [61] and this should be taken into account during the follow-up of these patients.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

References
