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Review

Platelet Function in Experimental Models of Liver Cirrhosis

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Abstract

Platelet function is commonly altered in liver cirrhosis. In an experimental model of liver cirrhosis, we have analyzed the mechanisms of defective platelet function, related to several alterations compatible with the existence of a hyperaggregatory state. These alterations are related to defective platelet calcium handling, specifically enhanced intracellular calcium release, which is evoked by agonists and an increased amount of calcium stored in the intracellular organelles. These alterations are manifested before the appearance of ascites, thus representing an early phase of the disease. Homocysteine plays a role in this enhanced platelet aggregation response, probably through the enhanced formation of reactive oxygen species, which can be prevented by folic acid pretreatment. Bile acids show a tendency to reduce calcium movements across platelet membranes which reduces this hyperaggregatory state, i.e., a characteristic of the non-ascitic phase of the disease. Chronic treatment with folic acid eliminates these alterations and helps in minimizing the risks associated with thrombotic events in cirrhosis.

Keywords

Cholestasis; Calcium; Thrombin; Liver cirrhosis; Nitric oxide; Oxidative stress

1. Introduction

Liver cirrhosis is the final stage of several diseases that affect the liver. The structural alterations caused by this disease were detected for the first time in 1760 by Morgani and in 1819 Laenec. They described the disease in a dead soldier, as "cirrhosis" (from the Greek kirrhos = yellow), due to the yellow granulations present in the liver [1]. This disease is characterized by an irreversible distortion of the hepatic architecture and high accumulation of fibrosis, which leads to functional failure at a systemic level. Liver cirrhosis appears in portal hypertension, cardiomyopathy, hepatopulmonary syndrome, coagulopathies, renal dysfunction with water and salt retention, which generate ascites and hyponatremia [2–3]. Cirrhosis can be considered a premalignant condition since there is a high risk of developing hepatocarcinoma, being the fifth most common cancer, causing 600,000 deaths per year worldwide [4]. Chronic alcohol consumption and hepatitis B and C viruses are the most frequent causes of cirrhosis. Less common causes include idiopathic hemochromatosis, Wilson's disease, autoimmune hepatitis, cystic fibrosis, non-alcoholic steatohepatitis, chronic obstructive cholestasis (biliary cirrhosis), obstruction of venous drainage, and hepatotoxicity [5–9].

1.1 Physiology of Platelets

The characteristics of platelets (shape, content of their granules, high density of adhesion receptors, and their ability to generate thrombin) are conducive for the formation of a stable clot under high flow conditions [10]. The contribution of platelets in hemostasis is different in arteries and veins. In the veins, being a low flow system, which allows the accumulation of activated coagulation factors and the local generation of thrombin, the platelet function is less prominent. In fact, the main cellular components of the venous thrombus are erythrocytes. In the case of arteries, the platelet function is essential, as being a high-flow system, there is a continuous wash out of coagulation factors. Platelets first plug the wound and provide a surface, where thrombin can generate fibrin. In addition, the endothelial cells aid in the recruitment of coagulation factors and the release of prostaglandin I2, nitric oxide (NO), and protein C. They inhibit the formation of thrombin and allow the action of coagulation factors V and VIIIa. The pathological formation of an arterial thrombus occurs in case of a disease or as a side effect of certain drugs where unjustified platelet activation occurs, producing an accumulation of platelets in a place where it is not needed. In general, platelets are activated by agonists whose receptors are expressed on the surface of platelets.

The platelets are the smallest and the second most abundant cell elements that circulate in the blood, representing a count in normal limits of 150–400 x 10^9 platelets/L. These cells are actual cellular fragments shed off from megakaryocytes (present in the bone marrow), which release about 10^{11} platelets a day into the bloodstream [11]. Platelets remain for a short time (10 days) in the bloodstream due to the absence of a nucleus [12]. They have a flattened disc morphology (2–4 x 0.5 μm). This, along with their small size, provides them an ability to constantly evaluate the integrity of the vessels, as they can cross the vessel wall. When a vessel is damaged, the platelets carry out a series of processes, namely, adhesion, activation, aggregation, generation of procoagulant substances, and retraction of the clot. The series of these events result in a clot that quickly occludes the site of the damaged vessel, preventing blood loss. Eventually this clot gets

dissolved when the damaged tissue has been restored, resulting in wound healing. Platelets express at least 10 different G proteins, with different functions, during their activation. There are also G protein signal regulators that inhibit platelet function. Given the complexity of normal platelet functions, it is not surprising that many defects acquired or inherited might contribute to platelet dysfunction, thereby increasing the risk of bleeding.

1.1.1 Primary and Secondary Hemostasis

It is necessary to distinguish between primary and secondary hemostasis. The first one acts first, as its name suggests and corresponds to the cellular compartment where platelets are involved. Secondary hemostasis corresponds to the plasma compartment containing the coagulation factors. These two concepts are interesting in the perspective of laboratory studies and are very interactive. Platelets are essential for directing the anchorage of coagulation factors since the activated platelets and surface phospholipids facilitate the generation of thrombin, but at the same time, thrombin is one of the most potent stimulators of platelets [13]. The other sub-processes involved in primary hemostasis after an injury of the vascular wall are as follows:

1. Exposure of the subendothelial matrix of the damaged vessel, represented mostly by the exposed collagen.

2. Platelet adhesion to the vascular wall. This is initiated by the von Willebrand factor (VWF) that joins or acts as a bridge via its A1 domain between the collagen and the GPIb/IX/V multiprotein complex. Red blood cells help the GPIb/IX/V complex by facilitating the contact between platelets and the vascular wall, as well as providing adenosin diphosphate (ADP). The Bernad-Soulier syndrome involves the GpIb deficit, thus preventing platelet adhesion to the vessel wall.

3. Platelet activation. It is a complex process, where the steps are interspersed.

3.1. Platelet aggregation starts when collagen contacts with platelet GPVI, which generates a chain of events involving other agonists. The activation of phospholipase C (PLC) occurs through different agonists such as thrombin, ADP, and thromboxane A2 (TxA2) that activate PLCβ through Gq as an intermediate and collagen that activates PLCγ2 through the protein tyrosine kinase GPVI. Besides this, there are some receptors involved such as PAR1 (receptor of activated protease 1, for thrombin), P2Y12 (receptor for ADP), TP (receptor for TxA2). The activation of PLC hydrolyzes membrane-bound phosphatidylinositol-4,5-biphosphate, producing inositol-1,4,5-triphosphate (IP3) and other secondary messengers required to release cytosolic calcium. This leads to the activation of integrins with different routes of action (CalDAG-GEF, Rap1-GTP, kindlin, and talin). The activation of talin, which binds to the αIIbβ3 domain of GPIIb/IIIa receptors, which are found in the platelet membrane, produces a conformational change in this protein that allows binding of its free end with fibrinogen. Bivalent binding to fibrinogen makes it possible for the platelets to bind with each other, thus facilitating the aggregation of platelets. Mutations in the GPIIb/IIIa protein cause a deficit in the aggregation giving rise to diseases such as Glanzmann's thrombasthenia, where platelets are unable to form stable aggregates to maintain hemostasis.

3.2. Platelet degranulation and release of substances such as TxA2 and microvesicles/granules: TxA2 is released via the action of the enzyme cyclooxygenase (COX) on arachidonic acid (AA), which is released from the platelet phospholipid membrane (this is the reason why the function of COX is abolished in patients taking aspirin or other nonsteroidal anti-inflammatory drugs). Out of

the δ-granules, ADP, serotonin, and calcium are released; and α-granules release GP (P-selectin, GMP–33, GPIIb/IIIa), VWF, fibrinogen, and coagulation factors. These substances together with the phospholipids of the platelet membrane are then exposed to the membrane.

3.3. The release of Ca^{2+} that occurs during platelet activation has been thought to occur in two steps:

3.3.1. Release of Ca^{2+} granules from the dense intracellular tubular system caused by IP3: This process is carried out with the help of the STIM1 protein.

3.3.2. Entry of Ca^{2+} from outside the cell through the plasma membrane: This process is carried out via STIM1-Orai1 protein.

4. Stabilization of the clot: Signal amplification occurs through ligands and G protein receptors that allow a stable platelet clot. These signals are produced through integrins and receptors whose ligands are located on the surface of adjacent platelets. The result is the generation of thrombin and a reticulated fibrin mesh where platelets are embedded. The local generation of thrombin secondary to platelet activation allows the platelet surface to offer phosphatidylserine, a place where the coagulation factors can bind. During this process, platelets also facilitate the migration of leukocytes around the tissue, as well as other inflammatory mediators that help in wound healing [10, 12].

2. Hemostasis in Cirrhosis

The liver plays a central role in the maintenance of hemostasis since it is the site of synthesis of the majority of proteins required for the regulation of coagulation and fibrinolysis. Therefore, it is logical to think that patients with cirrhosis present severe alterations in coagulation, triggering dysfibrinogenemia, hyperfibrinolysis, and thrombocytopenia. Among them, thrombocytopenia is the most frequent alteration in the final stages, which, in part, caused by portal hypertension, as 90% of the platelet pool is sequestered in the spleen (hypersplenism). This also occurs in the presence of antiplatelet antibodies or alterations in the metabolism of thrombopoietin [14–15]. Hemostasis in patients with cirrhosis is influenced by multiple contrasting variables, which may favor both the appearance of bleeding episodes and hypercoagulation states [16–17]. The platelet abnormalities described in cirrhosis include not only thrombocytopenia, but also the defects in the platelet aggregation, either due to hyper or hypo-response [18]. Therefore, in coagulation disorders, there may be an increase in the factors that promote bleeding with procoagulant alterations that induce the appearance of thrombosis. Among the latter, we can mention the decrease in the activity of some anticoagulation mechanisms and vascular stasis as a consequence of slowed down circulatory flow and impairments in the fibrinolysis and platelet activity [19].

The literature reports at least two types of coagulopathy, depending on the origin of liver cirrhosis [20]. Thus, it has been observed that patients with hepatic cirrhosis of cholestatic origin have a lower number of bleeding complications when compared with those in a similar clinical state but with the cirrhosis of viral or alcoholic etiology [21–23]. Although the origin of this alteration is not clear, the existence of a hypercoagulable state has been suggested in patients with primary biliary cirrhosis, due to a more efficient platelet function in these patients than in others [24]. In fact, the density of CD42b membrane platelet receptor is higher in cholestatic patients than in non-cholestatic patients [23]. As it is known, this antigen is a part of the GP Ib/V/IX adhesion platelet receptor that binds to von Willebrand factor (vWF) and it is internalized

after platelet activation. The presence of these binding sites allows the platelets to stop their movement in the arterial circulation and initiate their adhesion activation process and subsequent aggregation [25]. However, in patients with cirrhosis of alcoholic or viral origin, what has been described is just the opposite of this process, i.e., the existence of a serious defect in platelet adhesion [26].

In our previous studies [27–28], in a rat model of liver cirrhosis by bile duct ligation, several platelet alterations compatible with the existence of a hyperaggregatory state were demonstrated. Some of these alterations were also observed in a cholestatic group, which represented a phase prior to the development of overt liver cirrhosis. In general, these alterations are related to defective platelet Ca^{2+} handling, specifically to enhanced intracellular Ca^{2+} release that is evoked by thrombin and an increased amount of Ca^{2+} stored in the intracellular organelles, and are present before the appearance of ascites. The response to thrombin was significantly enhanced in platelets in BDL rats, both in the absence and presence of extracellular Ca^{2+}. When platelets were stimulated with thrombin in the absence of external Ca^{2+}, the elevation in $[Ca^{2+}]_i$ was caused by the Ca^{2+} release from the internal stores, mainly the endoplasmic reticulum, and our results indicated that thrombin-stimulated release of Ca^{2+} from the internal stores was clearly elevated in platelets from the cholestasis and BDL groups. When we used 2-APB, a cell-permeant IP3 receptor blocker, a reduction in the thrombin-induced Ca^{2+} release was observed. However, under these conditions, a significantly greater thrombin-evoked response was still observed in platelets from BDL rats, which suggests that this alteration is at least partly independent of the IP3-dependent pathway. Another explanation for the increased release of Ca^{2+} in platelets from BDL rats is that the amount of Ca^{2+} stored is greater. Indeed, when we induced extensive depletion of the intracellular stores with thapsigargin and ionomycin, it was found that the amount of Ca^{2+} accumulated into the intracellular compartments by platelets in the BDL rats was significantly greater than that of the controls. These results suggested that the increased Ca^{2+} release induced by thrombin from platelets in the BDL rats may be related to elevated accumulation of Ca^{2+} in the intracellular stores.

We also analyzed the so-called capacitative calcium entry, which is known to play a central role in calcium signaling [29]. Essentially, calcium entry is regulated by filling of the calcium stores, so when calcium is depleted or discharged from the stores, calcium entry from the external environment is promoted. Thus, when thapsigargin was used to deplete the internal stores, we observed a slow and sustained increase in $[Ca^{2+}]_i$ level that was greater in platelets of the cholestasis and BDL groups compared with the controls. However, when Ca^{2+} was added again, it produced a rapid increase in $[Ca^{2+}]_i$ indicative of Ca^{2+} entry, but the amount of Ca^{2+} that entered the cells was significantly lower in the platelets from the BDL group. A tendency to lower Ca^{2+} entry was also observed in the cholestasis group but it did not reach statistical significance. Additionally, the activity of both calcium pumps, i.e., PMCA and SERCA, was found to be significantly increased in the BDL group, not in the cholestasis group. In conclusion, chronic bile-duct ligation alters intracellular Ca^{2+} homoeostasis in platelets, such that an enhanced Ca^{2+} release is evoked by thrombin. This may be due to the increased amount of Ca^{2+} stored in the intracellular organelles and secondary to the enhanced activity of SERCA. These alterations occur during the cholestasis phase and can be evident much before cirrhosis has completely developed.

Although it is not known with certainty what mechanisms cause the onset and maintenance of hemostatic disorder in cirrhosis, several studies suggest that high levels of homocysteine (Hcy) in

plasma could be an effective inducer of fibrogenesis of the liver since they interfere with the structure and function of several proteins (N-homocysteinylation) to induce the expression of procollagen type I [16, 30]. The latter also alters the metabolism of plasma fatty acids and lipid membranes, leading to changes in membrane receptors [31]. In turn, Hcy is easily oxidized in the plasma, mainly as a result of its auto-oxidation, generating superoxide anions (O–2) and hydrogen peroxide (H_2O_2) (32). These molecules produce oxidative stress, endothelial damage, and inflammatory processes, which contribute to platelet activation [31]. In these patients, hypercoagulation can manifest in two ways. One, in the form of deep vein thrombosis or pulmonary embolism, with an incidence ranging from 0.5 to 1% [33]. The second one, in a more overlapping manner, with occult microthrombosis that, over time, leads to porto-pulmonary hypertension or decompensated hepatic atrophy [34–36].

Several studies have shown that there is an increase in plasma Hcy in patients with cirrhosis of any etiology, as well as in non-cirrhotic liver patients. Therefore, hyperhomocysteinemia (HHcy) was associated with the course of liver disease and damage to the kidney, being more pronounced in the advanced stages of cirrhosis, since the liver loses its integrity as an organ, causing a macro imbalance and micronutrients [16, 37–38]. Therefore, in cirrhotic patients, HHcy, in most cases, is due to poor absorption of B vitamins, which are required for the metabolism of Hcy. However, drugs can also interfere with the metabolism of these vitamins and produce HHcy [39]. It is for this reason that a cirrhotic patient is normally provided with a diet rich in B vitamins (folic acid, B6, and B12) and proteins preferably of plant origin because they are poor in aromatic amino acids, ammonia, and mercaptans [40]. The pathological mechanism by which HHcy promotes vascular atherothrombotic alterations is not completely known. Several studies have shown that Hcy, being rapidly self-oxidized in plasma, forms mixed disulfides and Hcy thiolactone, generating powerful reactive oxygen species [41–42]. These reactive oxygen speciescause endothelial damage and decrease in the production of NO, thus damage the platelet activity and induce its activation, thereby exerting a powerful atherogenic effect [42–44]. Experiments performed in mice with HHcy have shown an increased sensitivity to experiencing thrombosis, so that the thrombotic occlusion of the carotid artery occurs 50% more rapidly in heterozygous hyper-homocysteinemic mice than in the normal mice. Several studies have shown that HHcy stimulates platelet aggregation, along with an increase in the synthesis of thromboxane A2 (Minno, et al., 1993), activation of factors V and XII [45], increased tissue factor (FT) activity that promotes coagulation in the absence of thrombin [46], and an increase in the secretion of von Willebrand factor. On the other hand, Hcy also inhibits important physiological anticoagulant pathways, such as the activation of protein C and the expression of thrombomodulin on the endothelial surface [48]. In addition, alterations have been found in the binding with antithrombin III and the reduction of tissue plasminogen activator binding (t-PA) [48]. As we know, Ca^{2+} ion is very important for platelet activation, but the mechanism by which Hcy activates Ca^{2+} entry is poorly known. The treatment of platelets with Hcy increases the levels of intracellular Ca^{2+} and promotes the activation of protein kinase C [49], contributing to the association of cytosolic phospholipase A2 (cPLA2) with the platelet membrane. Consequently, Hcy (in a dose-dependent manner) increases the phosphorylation of cPLA2 and activation of platelets through p38 MAPK and these effects are closely related to the increase of intracellular Ca^{2+}, leading to an increase in AA and formation of TXB2 [49–51]. The greater Ca^{2+} flow in platelets is caused by the capacitive input (ECC), that is, by the emptying of intracellular Ca^{2+} deposits to produce a signal that opens channels in the plasma membrane, which induces an

increase in the cytosolic Ca^{2+} and filling of the deposits. This finally closes the Ca^{2+} channels. In this way, the signal that opens the Ca^{2+} channels is not the activation of the receptor nor the generation of IP3, per se, but the emptying of the Ca^{2+} deposits themselves. Earlier studies performed in an experimental model of cirrhosis in BDL rats (bile duct ligation) found an alteration in the ECC, where the release of Ca^{2+} from the intracellular stores caused by the thapsigargin was higher in the group with BDL and with a notable minor ECC, with respect to its control [52]. Whereas after an acute incubation with Hcy, no change was observed, possibly the greater effect on the aggregation was not at the level of a specific stimulation of Ca^{2+} receptors, but rather through certain secondary messengers or mediators, as is the case of nitric oxide, whose reduction results in a moderate increase in sensitivity to the agonist [51–53]. In this model of biliary cirrhosis in rats, it was also seen that Hcy, acutely, was able to increase the area and maximum platelet aggregation peak but was not responsible for the increased expression of P-selectin, because the receptor was already over-expressed in BDL animals. Further, after chronic treatment with folic acid, the area of aggregation was decreased by 57.5% with respect to its control group without treatment. It was also able to decrease the expression of P-selectin. This indicates that Hcy has a physiological role in platelet aggregation and is fundamental for the proper functioning of platelets [52–53]. It is also known that folic acid deficiency in cirrhosis can produce an increase in the activity of tissue factor (TF) by the stimulation of endotoxins derived from macrophages, which also contributes to pro-coagulant activation (PCA). The platelet cell surface is more suitable for the catalytic activity of FT or improves the binding capacity of FT once it has been expressed on the cell surface, thus increasing the thrombogenic capacity of platelets [31, 54]. There are a large number of studies on the inhibitory effect of folic acid on platelet function and the existence of decreased plasma levels in liver disease. These data indicate that a diet supplemented with folic acid can improve platelet function in cirrhosis and decrease aggregability. However, a study by Marsillach et al. [55] showed that folic acid supplementation in cirrhotic rats induced an increase in fibrosis, concluding that an overdose of this vitamin in patients with liver disease may be contraindicated. Further studies are necessary in order to conclude the effect of folic acid supplementation in cirrhosis. However, in cirrhotic patients, the hypoaggregability produced by the treatment with folic acid could also enhance bleeding time and the risk of digestive hemorrhage due to the rupture of varicose veins triggered by portal hypertension. In summary, in cirrhosis, the normal homeostatic reserve capacity to control bleeding and thrombosis events is lost with the predilection toward bleeding or thrombosis dependent on the individual and the precipitant [17].

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Competing Interests

The authors have declared that no competing interests exist.

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