Novel functions of platelets in the liver

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Abstract
Platelets contain not only proteins needed for hemostasis but also many growth factors that are required for organ development, tissue regeneration, and repair. Thrombocytopenia, which is frequently observed in patients with chronic liver disease (CLD) and cirrhosis, is due to various causes, such as decreased thrombopoietin production and accelerated platelet destruction caused by hypersplenism; however, the relationship between thrombocytopenia and hepatic pathogenesis and the role of platelets in CLD is poorly understood. Thus, in this paper, the experimental evidence for platelets improving liver fibrosis and accelerating liver regeneration is summarized and addressed based on studies conducted in our laboratory and current progress reports from other investigators. Platelets improve liver fibrosis by inactivating hepatic stellate cells to decrease collagen production. The level of intracellular cAMP is increased by adenosine through its receptors on hepatic stellate cells, thereby resulting in inactivation of these cells. Adenosine is produced by degradation of adenosine nucleotides, which are stored in abundance within the dense granules of platelets. The regenerative effect of platelets in the liver consists of three mechanisms: a direct effect on hepatocytes, a cooperative effect with liver sinusoidal endothelial cells, and a collaborative effect with Kupffer cells. Based on these experiments, a clinical trial suggested that the increase in platelets induced by platelet transfusion improved liver function in patients with CLD in a clinical setting. We highlight the current knowledge concerning the role of platelets in CLD and expect to open a novel avenue for application of these clinical therapies to treat liver disease.

Introduction
Chronic liver disease (CLD) is a major cause of mortality and morbidity in many countries, and cirrhosis is the end stage of CLD. Cirrhosis carries a poor prognosis and an increased risk of carcinogenesis. Liver fibrosis represents the consequence of a sustained wound-healing response to chronic liver injury induced by a variety of causes, including viral infection, alcohol abuse, autoimmune disorders, drug use, cholestasis, and metabolic diseases. Currently, liver transplantation is still the only curative approach for end-stage cirrhosis, but this process is associated with serious problems, such as graft shortage in living-donor liver transplantation, surgical complications, organ rejection, and high cost. Although liver fibrosis has traditionally been regarded as an irreversible process, recent reports indicate that even advanced fibrosis may be reversible. Based on these studies, novel treatments have been developed to treat patients with cirrhosis.

Thrombocytopenia is a common complication of CLD and is due to various causes, including decreases in thrombopoietin (TPO) production, increases in platelet destruction with splenomegaly, and an inability of bone marrow to perform hematopoiesis. Therefore, thrombocytopenia is thought to be intimately related to the pathogenesis of CLD and cirrhosis.

The effect of thrombocytopenia on liver damage and the exact mechanisms that lead to thrombocytopenia in CLD and cirrhosis are still unclear, and further study is required. In our previous studies, we revealed that platelets play a crucial role in promoting liver regeneration.

Platelets (anuclear blood cells) are derived from megakaryocytes (MKs), which contain not only proteins needed for hemostasis but also many growth factors that are required for tissue regeneration and repair.

We also reported that platelets have a preventive effect on the progression of liver fibrosis in vitro and in vivo and that the increase in platelets induced by platelet transfusion can improve the liver function of patients with CLD and cirrhosis in a clinical setting.

In addition, it was reported that splenectomy, which is a platelet increment therapy, contributes to improvement of liver function.

However, there are many contradictory reports that platelets have harmful effects on liver fibrosis.
The aim of this review is to summarize the clinical and experimental studies that have broadened our understanding of the role of platelets in CLD.

**Platelets**

Platelets are derived from MKs. MKs are derived from multipotent hematopoietic stem cells toward MK progenitors. Mature MKs produce platelets by cytoplasmic fragmentation, which occurs through a dynamic and regulated process called proplatelet formation; these proplatelets consist of long pseudopodial elongations that break in the blood flow. Recently, it has been reported that IL-1α induces thrombopoiesis through MK rupture in response to acute platelet needs. Platelets are discoid and have anucleate structures that contain a large number of secretory granules, alpha granules, dense granules, and lysosomal granules, of which three types of secretory granules are recognized.

Each granule contains secretory substances, such as platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), serotonin, adenosine diphosphate (ADP), adenosine tri-phosphate (ATP), epidermal growth factor (EGF), transforming growth factor-β (TGF-β), and sphingosine 1-phosphate (SIP). Platelets are activated by various types of stimulation and release active substances from their granules.

Opposite effects have been reported: the positive ones include processes such as hemostasis, wound healing, and tissue regeneration, whereas the negative effects of platelet degranulation include inflammation, malignancy and immune response. It has been reported that platelets accumulate in the liver under certain pathological conditions, such as ischemia/reperfusion, cirrhosis, cholestasis, viral hepatitis, and the residual liver after hepatectomy.

**Platelets and liver fibrosis**

Currently, liver fibrosis is known to be part of a dynamic process of continuous extracellular matrix (ECM) remodeling in the setting of chronic liver injury; this process leads to excessive accumulation of several extracellular proteins, proteoglycans, and carbohydrates. Among the cellular populations in the liver, hepatic stellate cells (HSCs) have been reported to have the most involvement in liver fibrosis through production of large amounts of ECM and secretion of TGF-β, which appears to be a key mediator of liver fibrogenesis. In the normal liver, HSCs reside in the space of Disse, and their primary function is storage of vitamin A and other retinoids. In addition, HSCs are now well established as the key cellular element that is involved in the development of liver fibrosis. In response to liver injury, HSCs undergo morphological and functional transdifferentiation, converting from vitamin A-storing star-like cells into contractile myofibroblastic cells; this process is called activation. Ikeda et al. reported that human platelets contribute to suppression of both HSC activation and type I collagen production via a cyclic AMP signaling pathway in vitro (Fig. 1). The level of intracellular cAMP is increased by adenosine through its receptors on HSCs. Large amounts of adenosine around HSCs are produced by degradation of adenosine nucleotides, such as ADP and ATP, which are stored in abundance within the dense granules of platelets. Quiescent HSCs that are inactivated by adenosine may have a decreased ability to produce TGF-β and secrete ECM. In addition, platelet-derived HGF plays a critical role in suppression of type I collagen gene expression in cultured HSCs. HGF has also been reported to attenuate liver fibrosis through suppression of HSC activation and hepatic TGF-β expression. These findings indicate that platelets can play a crucial role in suppression of liver fibrogenesis via inhibition of HSC activation. Because human platelets contain a smaller amount of HGF than rodent platelets, it is unclear whether the mechanisms observed in rodents are applicable to humans.

Thrombopoietin is the most important growth factor in the regulation of MK development and platelet production. Several promising novel agents that stimulate TPO receptors and increase platelet levels, such as eltrombopag and romiplostim, are currently in development for the treatment of thrombocytopenia in patients with CLD and cirrhosis. The ability to increase platelet levels could significantly reduce the need for platelet transfusions and facilitate the use of interferon-based antiviral therapy and other treatments in patients with liver disease. Recently, it was reported that the increase in platelets induced by TPO administration could improve liver fibrosis, even in subjects with CLD and cirrhosis, in experimental studies. Dimethyltritosamine was administered three times a week for three weeks to induce liver fibrosis in rats. Five days after administrating TPO intravenously, 70% hepatectomy was performed, and the liver fibrosis was compared 24 h after the hepatectomy. The increase in platelets inhibited the activation of HSCs and reduced the fibrotic area of the cirrhotic liver; these effects were diminished by administration of antiplatelet serum. Carbon tetrachloride (CCL₄) was
administered twice a week for 8 weeks to induce liver fibrosis in mice. TPO was administered intraperitoneally once a week from 5 to 8 weeks during the experiment. By administering TPO, liver fibrosis was decreased. Although the precise mechanisms that relate the increase in platelets and the liver anti-fibrotic effect are still unclear, one reason may be that platelets enhanced the expression of HGF by approximately 14%.11 whereas the matrix metalloproteinase 9 (MMP9) was enhanced by approximately three times, thereby stimulating fibrolysis and decreasing profibrotic growth factor TGF-β.13 MMPs such as MMP-8, MMP-9, and MMP-13 possess the ability to degrade the extracellular matrix via the breakdown of collagen type I.40 MMP-9 may indirectly contribute to fibrolysis by accelerating HSC apoptosis.35 In the murine bile duct ligation model, thrombocytopenia exacerbates liver fibrosis and platelets have an anti-fibrotic role by suppressing type I collagen expression via the HGF–Met signaling pathway.34 Recently, Takahashi et al. reported that transfused human platelets improved liver fibrosis in severe combined immune deficiency (SCID) mice induced by CCL4.42 An increase in murine HGF and a decrease in TGF-β were observed in the liver.42 Although the precise mechanism that relates the increase in platelets and liver fibrolysis remain unclear, one reason is that platelets enhanced the expression of HGF without an increase in the expression of pro-fibrotic growth factors derived from platelets, such as TGF-β and PDGF, in the cirrhotic liver in rodent models.11,35

Platelets and liver regeneration

Liver regeneration is performed by proliferation of hepatocytes, biliary epithelial cells, liver sinusoidal endothelial cells (LSECs), Kupffer cells, and hepatic stellate cells, all of which proliferate to rebuild the lost hepatic tissue.43 Intercellular interactions via many growth factors and cytokines, including HGF, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), transforming growth factor-α (TGF-α), and EGF, play important roles. Each growth factor and cytokine leads to subsequent activation of downstream transcription cascades, which is associated with the transitions of quiescent hepatocytes into the cell cycle and progression beyond the restriction point in the G1 phase of the cycle.43 Several transcription factors are known to have functions in this process: nuclear factor-kappa B (NF-κB), activator protein 1 (AP-1), CCAAT/enhancer binding protein-b (C/EBPb), extracellular signal-regulated kinase 1/2 (ERK 1/2),44 signal transducer and activator of transcription 3 (STAT3), and phosphatidylinositol-3-kinase (PI3 K)/Akt.45 The current study focused on the TNF-α/NF-κB, IL-6/STAT3, and PI3 K/Akt pathways as the three major cascades in which platelets exert their effects during the process of liver regeneration.

The TNF-α/NF-κB pathway is activated within 30 min after hepatectomy, and the activation usually lasts 4–5 h. NF-κB is found in almost every cell, including hepatocytes and non-parenchymal cells. It is usually inactivated by Inhibitor of NF-κB (IκB), which binds to the p65 subunit.46 After NF-κB is stimulated by TNF-α and activated by removal of IκB from the p65 subunit, it migrates to the cell nucleus and binds to the promoter of cyclin-D1, which regulates the G0/G1-to-S-phase transition.47 STAT3 is activated 1–2 h after hepatectomy and lasts 4–6 h. STAT3 is phosphorylated after being stimulated by IL-6 and translocates to the nucleus, where it promotes the expression of cyclin-D1 and p21 to control the progression of the cell cycle.48 Haga et al. reported that hepatocytic mitosis was significantly suppressed in STAT3- knockout mice after partial hepatectomy.49 The PI3 K/Akt pathway was activated shortly after hepatectomy. The pathway is stimulated by HGF, IL-6, TNF-α, TGF-α, or many other signaling molecules.50 C-met is one of the tyrosine kinase receptors on the surface of hepatocytes that binds with HGF, and HGF/c-met signaling activates PI3 K, which recruits Akt to the site of membranes and, subsequently, phosphorylates Akt.51 Phosphorylation of Akt results in activation of glycogen synthase kinase (GSK) 3b, thus resulting in accumulation of β-catenin and cyclin-D1 in the nucleus, and induces DNA synthesis and cellular mitosis of hepatocytes.52

However, it has never been directly demonstrated that release of growth factors from platelets is responsible for platelet-mediated stimulation of liver regeneration in vivo.53,54

Mechanisms for the promotive effect of platelets in liver regeneration

The direct effect of platelets. Platelets accumulate in the thrombocytotic liver within 5 min after hepatectomy, and a twofold increase in platelets is observed in the liver in comparison with pre-hepatectomy levels.55 Electron microscopy has revealed that platelets translocate from the sinusoidal space into the space of Disse and have direct contact with hepatocytes.56,57 Endo et al.56 reported a similar phenomenon, in which platelets accumulate in the liver and a large number of platelets are detected in the space of Disse in response to the administration of lipopolysaccharide (LPS), interleukin-1, and TNF-α. Intravital microscopy reveals that although a few platelets flow rapidly in the liver sinusoids before partial hepatectomy, a significant number of platelets accumulate in the liver sinusoids, and the platelets flow slowly with rolling and adhering after hepatectomy.58 These results suggest that platelets accumulate in the liver within a few minutes after hepatectomy and provide signals for rapid hepatocyte proliferation through direct contact with hepatocytes. A co-culturing chamber system was used to clarify the role of the direct contact of platelets with hepatocytes. Platelets and hepatocytes are separated by a permeable membrane.10 Direct contact between platelets and hepatocytes triggers the release of soluble mediators from platelets, such as HGF, IGF-1, and VEGF, which leads to hepatocyte proliferation (Fig. 2).

The mechanism that underlies the direct effect of platelets can be explained. Platelets accumulate in the liver immediately after hepatectomy, translocate from the liver sinusoids to the space of Disse and release growth factors, such as HGF, IGF-1, and VEGF, through direct contact with hepatocytes. Growth factors stimulate the initiation of hepatocyte mitosis, which eventually promotes liver regeneration. Human platelet does not include most of the HGF; thus, IGF-1 is considered to be the most important mediator for liver regeneration in human platelets. Recently, it has been reported that RNA transfer mechanism as an additional contributor to platelet-mediated liver regeneration.57

The collaborative effect with Kupffer cells. Kupffer cells play a role in the liver as macrophages that act against bacteria, endotoxins, and microbial debris derived from the
gastrointestinal tract. Kupffer cells produce important cytokines that have a stimulatory influence on hepatocyte proliferation after hepatectomy. One of the most important events following hepatectomy is an increase in the plasma levels of TNF-α. An experiment using an antibody against TNF-α demonstrated a significant reduction of hepatocyte proliferation, and mice lacking the TNF-α receptor exhibit severe impairment in liver regeneration after hepatectomy. Activation of the TNF-α receptor increases the hepatic expression of the NF-κB in both hepatocytes and non-parenchymal cells and is followed by production and release of IL-6 from Kupffer cells. Kupffer cells are considered to be the most important source of both TNF-α and IL-6. Kupffer cell-depleted mice fail to exhibit increases in TNF-α and IL-6 to levels that are equivalent to those in mice with Kupffer cells after hepatectomy. The interrelationship between platelets and Kupffer cells has been well studied using ischemia/reperfusion models. A triangular interaction among platelets, Kupffer cells, and leukocytes has been demonstrated as the major mechanism of injury. Rats depleted of Kupffer cells were subjected to ischemia/reperfusion, which suppressed platelet adhesion in sinusoids, and, as a consequence, there was attenuation of sinusoidal perfusion failure and endothelial damage. Tamura et al. reported that platelets adhered to Kupffer cells at the early period of ischemia/reperfusion and that the platelets that adhered Kupffer cells were involved in apoptosis of hepatocytes and the mechanism of hepatic ischemia/reperfusion injury. Nakamura et al. described different characteristics for platelets and Kupffer cells. The authors reported that direct cellular interactions between platelets and Kupffer cells play an important role in platelet migration to the space of Disse after administration of LPS. It is therefore likely that platelets are activated by direct contact with Kupffer cells, and Kupffer cells are stimulated by direct contact with platelets. The collaborative effect of platelets and Kupffer cells on liver regeneration is thought to occur after hepatectomy, when activated Kupffer cells induce accumulation and activation of platelets in the liver, and the functions of Kupffer cells are enhanced by the accumulated platelets. Liver regeneration is promoted by the direct effect of growth factors released from platelets and by the paracrine effect of Kupffer cells, which is enhanced by the platelets (Fig. 2).

The cooperative effect with liver sinusoidal endothelial cells. Sinusoidal cells account for 70% of LSECs. The construction of a thin and continuous layer of sinusoidal endothelium forms a structural barrier that separates the hepatic parenchyma from blood constituents that pass through the liver. LSECs enable contact between circulating blood and hepatocytes and help exchange various soluble macromolecules and nanoparticles, such as lipoproteins. LSECs are involved in liver regeneration and produce immune-regulatory and pro-inflammatory cytokines, including HGF, IL-1, IL-6, and interferons. The elevation of the IL-6 concentration after hepatectomy is an important component of the early signaling pathways in liver regeneration, and IL-6 activates the acute phase of protein synthesis by hepatocytes as a part of the overall inflammatory response. The plasma IL-6 concentration increases after hepatectomy from 6 h, with a peak by 24 h. IL-6 binds to the receptor on hepatocytes, which leads to phosphorylation of STAT3 monomers. The relationship between platelets and LSECs is well documented in ischemia/reperfusion models. In contrast, no studies have focused on the relationship between platelets and LSECs in liver regeneration. The role of platelets in liver regeneration in relation to LSECs was evaluated using co-culturing chamber systems. These studies clarified that platelets increase the proliferation of LSECs and induce IL-6 release from LSECs, and IL-6 derived from LSECs accelerates DNA synthesis in hepatocytes. Direct contact between platelets and LSECs was required for IL-6 excretion. In addition, the high level of sphingosine-1-phosphate (S1P) in platelets plays an important role in IL-6 secretion. S1P is a lipid mediator that regulates diverse biological processes, including proliferation, migration, and cytoskeletal reorganization. S1P is excreted in large amounts from activated platelets and interacts with endothelial cells under the conditions of critical platelet-endothelial interactions, such as thrombosis, angiogenesis, and atherosclerosis.
The cooperative effect of platelets with LSECs on liver regeneration occurs when direct contact between platelets and LSECs induces S1P release from platelets, which subsequently induces excretion of IL-6 from LSECs. IL-6 derived from LSECs promotes hepatocyte proliferation through the STAT3 pathway (Fig. 2).

**Effect of platelet transfusion on chronic liver disease and cirrhosis.** Platelet transfusion is an established therapy for thrombocytopenia, with well-known benefits and complications. The results suggest that both endogenous and exogenous platelets may play a role in the improvement of liver function, although whether platelet transfusion is appropriate for CLD patients with thrombocytopenia is still unclear, even in the published guidelines for platelet transfusion.71 Based on these animal experiments, clinical trials were performed. Maruyama et al. recently reported on a clinical trial to investigate whether platelet transfusion improves liver function in patients with CLD and cirrhosis (Child-Pugh class A or B) who all presented with thrombocytopenia (platelet counts between 50,000 and 100,000/μL). The subjects received 10 units of platelet concentrate once per week for 12 weeks. One and three months after the last transfusion, significant improvements in serum albumin were observed (Fig. 3). Serum cholinesterase improved for 9 months after the last transfusion. Serum hyaluronic acid, which represents liver fibrosis, exhibited a tendency to improve after the last transfusion. This clinical trial was a non-controlled, non-randomized study, in which only 6 patients were eventually analyzed.14

**Limitations of platelet increment therapy for CLD and cirrhosis**

Platelet increment therapies, such as administration of a TPO receptor agonist, splenectomy, and platelet transfusion, have been reported to have adverse effects, although they have ameliorating effects for CLD and cirrhosis.39,72,73 Portal vein thrombosis has been observed among patients who received eltrombopag or splenectomy.39 In addition, operative complications of splenectomy include hemorrhage, infection, and injury to the pancreatic tail.72 In platelet transfusion, platelets are frequently activated and may induce inflammatory reactions and unexpected side-effects, including febrile non-hemolytic reactions and acute lung injury.73 There are some reports regarding the detrimental effects of platelets on CLD and cirrhosis. Zaldivar et al. provided evidence that chemokine (C-X-C motif) ligand 4 (CXCL4), which is known as a platelet-derived factor, modulated liver fibrosis in animal models of chronic liver injury in vivo, although direct release of CXCL4 by platelets was not demonstrated.16 In addition, the proliferation and chemotactic migration of HSCs was significantly enhanced by CXCL4 without synthesis of collagen and expression of TGF-β in vitro.16 As one of the side-effects of platelet increment therapy, promotion of liver carcinogenesis should be considered because of the high risk of HCC in patients with CLD and cirrhosis.3 Carr et al. reported that patients with thrombocytosis (platelet levels > 400 × 109/L) had larger HCC sizes and better liver function than patients with platelet levels in the normal range.74 Sitia et al. demonstrated that antiplatelet drugs reduced the development of HCC in a mouse model of chronic hepatitis B virus infection, although this approach was not effective in the carbon tetrachloride-induced cirrhosis model.75 These findings indicate that platelets might have a promotive effect on liver carcinogenesis. However, it has been reported that TPO itself had no proliferative effect on HCC in vitro and in vivo experiments.76 Currently, two TPO-R agonists, eltrombopag and romiplostim, have been approved for treatment of chronic immune thrombocytopenic purpura. Ertrombopag is a small-molecule, non-peptide TPO-R agonist, whereas romiplostim is a peptide TPO-R agonist that is composed of an IgG Fc fragment.77 The efficacy of eltrombopag in hepatitis C virus-infected patients with thrombocytopenia before the initiation of pegylated interferon and ribavirin therapy has previously been reported.38 In contrast to TPO, eltrombopag does not activate the PI3K/Akt pathway.78 Another report demonstrated that eltrombopag has no proliferative effect in myelodysplastic syndromes and AML patients but rather inhibits the proliferation of the leukemia cell line.79 Thus, eltrombopag would be secure in HCC, and use of this type of TPO-R agonist can be anticipated as a novel treatment for liver disease.

**Summary and future work**

This review discussed the current evidence for platelets improving liver fibrosis and promoting liver regeneration. There is significant evidence that platelets play a role in improving fibrosis. ATP and ADP inside platelets are degraded by HSCs, and adenosine is incorporated into the HSCs. Cyclic AMP is increased by adenosine, ADP inside platelets are degraded by HSCs, and adenosine is incorporated into the HSCs. Cyclic AMP is increased by adenosine, and HSCs become inactivated by cyclic AMP. There are three different mechanisms of liver regeneration induced by platelets: a direct effect on hepatocytes; cooperation with Kupffer cells; and a cooperative effect with LSECs. Therefore, platelet therapy, that is, platelet transfusion and TPO receptor agonist administration, could open a new avenue to develop novel strategies for treatment of liver diseases for which there is currently no effective treatment except transplantation.

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