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Platelet and Liver Regeneration

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1. Introduction

Platelets are the smallest structures in circulating blood and have a convex disc construction with an equatorial diameter of 2-3 microns and have no nucleus. They are derived from megakaryocytes in the bone marrow. Following their normal life span of 8-10 days, they are removed from the circulation when passing through the spleen. Platelets have three types of secretory granules, i.e., alpha-granules, dense-granules, and lysosomal granules in the cytosol (Fig. 1). Each granule contains a specific mix of soluble factors, such as platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), serotonin, adenosine diphosphate (ADP), adenosine tri-phosphate (ATP), epidermal growth factor (EGF), and transforming growth factor beta (TGF-beta) (Blair et al., 2009; McNicol & Israels., 1999; Polasek et al., 2005). After being activated by physiological substances such as thrombin, collagen, thromboxane A2 (TXA2), epinephrine, and platelet-activating factor (PAF), or by non-physiological substances such as divalent cationophores and phorobol esters, platelets release these biologically active substances that exert various effects depending on the specific context (Holmsen, 1989; Suzuki H et al., 1992; Broos et al., 2011)

The main physiological role for circulating platelets is hemostasis when a vessel is injured (Holmsen, 1989). This process involves rapid adhesion of the platelets to the exposed subendothelium followed by platelet aggregation which culminates in the formation of a platelet plug that temporarily seals off the injured vessel walls. As they undergo this process, platelet activation leads to exocytosis of granular substances, release of newly synthesized mediators, and discharge of membrane-bond trans-cellular signaling molecules (Holmsen, 1989; Broos et al., 2011). Numbers of the various kinds of mechanisms facilitate platelet participation in other physiological or pathological process including inflammation (McNicol et al., 2008), malignancy (Mehta, 1984; Nash et al., 2002), immune response (Elzey et al., 2005; Sowa et al., 2009; Klinger & Jelkmann 2002; Sprague et al., 2008), wound healing (Mazzucco et al., 2010; Ranzato et al., 2009; Rozman & Bolta., 2007; Yamaguchi et al., 2010), and tissue regeneration (Radice et al., 2010; Dugrillon et al., 2002; Hartmann et al., 2010; de Vos et al., 2010; Rodeo et al., 2010).

Platelets have been reported to accumulate in the liver under some kinds of pathologic conditions, such as ischemia/reperfusion injury (Khandoga et al., 2003, 2006; Pak et al., 2010), liver cirrhosis (Zaldivar et al., 2010), cholestatic liver (Laschke et al., 2008) and viral

hepatitis (Lang et al., 2008). Furthermore, platelets flow out slow, with rolling and adhesion in the liver sinusoids, under stressed situations such as ischemia/reperfusion injury (Nakano et al., 2008). Previous works on such conditions have focused on platelets as producers of inflammatory cytokines and therefore being pro-inflammatory (Pereboom et al., 2008). However, recent clinical and basic studies have revealed other ways in which they affect liver biology and pathology.

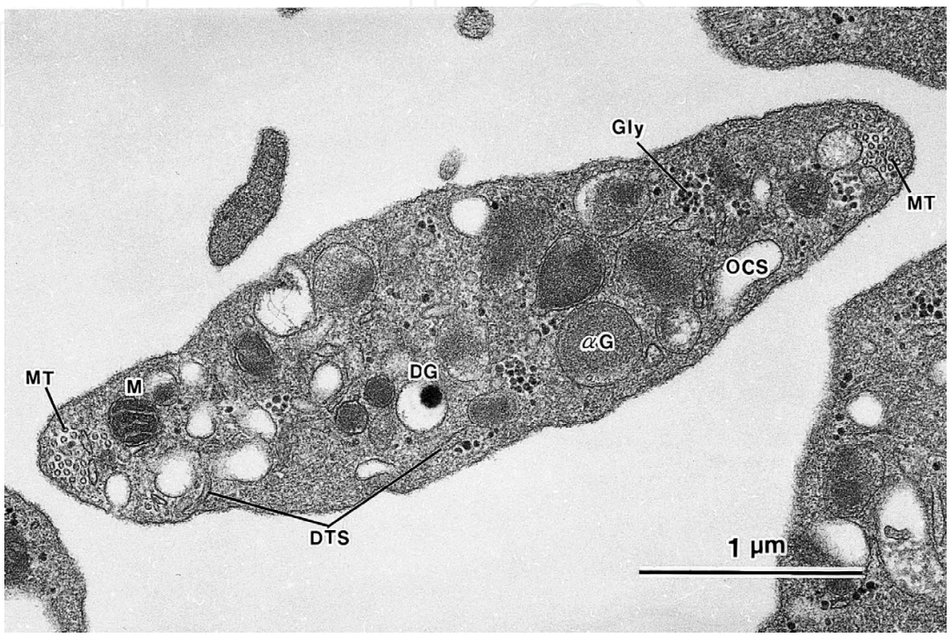


Fig. 1. Platelet ultrastructure. Transmission electron microscopic representation of a human platelets; the microtubules (MT), open cannalicular system (OCS), dense tubular system (DTS), mitochondria (M), alpha-granules (αG), dense granules (DG), and glycogen particles (Gly) are indicated. This electromicrograph is produced by kind permission of Dr. Hidenori Suzuki, Division of Morphological and Biomolecular Research, Graduate School of Medicine, Nippon Medical School.

In clinical studies, Marubashi et al. reported that there was a positive correlation between graft size and post-transplant thrombocytosis (Marubashi et al., 2006). Alkozai et al. described that a peri-operative low platelet count after partial hepatectomy was a predictor of delayed postoperative recovery of liver function and was associated with an increased risk of post-operative mortality (Alkozai et al., 2010). Kim et al. stated that total amount of platelet transfusion was positively associated with graft regeneration (Kim et al., 2010). In basic research, Lesurtel et al. showed that platelet-derived serotonin mediated liver regeneration in mice (Lesurtel et al., 2006). Nocito et al. demonstrated that platelets and platelet-derived serotonin promoted tissue repair after normothermic hepatic ischemia in mice (Nocito et al., 2007). In addition, we have obtained several types of evidence for platelets promoting liver regeneration using different experimental models of liver dysfunction in small and large animals.

In this chapter, we describe our evidence for platelets in promoting liver regeneration. Furthermore, we explain three different mechanisms i.e., 1) a direct effect on hepatocytes, 2)

a cooperative effect with liver sinusoidal endothelial cells (LSEC), and 3) a collaborative effect with Kupffer cells.

2. Growth factors, cytokines, and signal transduction related to platelets' effect on liver regeneration

Liver regeneration occurs by proliferation of all of the existing mature cellular populations including hepatocytes, biliary epithelial cells, LSEC, Kupffer cells, and hepatic stellate cells. Of these, hepatocytes are the first cells to proliferate (Malik et al., 2002); they usually replicate once or twice following partial hepatectomy and return to the quiescent state. The kinetics of cell proliferation differ between species, the peak of DNA synthesis in hepatocytes usually being at 24 hours in rats but at 36 hours in mice (Michalopoulos & DeFrances., 1997; Michalopoulos, 2010; Fausto et al, 1995, 2000). Intercellular interactions mediated by many growth factors and cytokines, including HGF, tumor necrosis factor-alpha (TNF-apha), interleukin-6 (IL-6), transforming growth factor-beta (TGF-beta), EGF etc. appear to play important role in this process. Each growth factor leads subsequent activation of downstream transcription cascades that drive transition of the quiescent hepatocytes into the cell cycle and ensure progression beyond the restriction point in the G1 phase of the cycle. Several transcription factors are involved, and nuclear factor-kappa B (NF-KB) (Tewari et al., 1992; Cressman et al., 1994; FitzGerld et al., 1995), activator protein 1 (Ap-1) (Stepniak et al., 2006), CCAAT/enhancer binding protein-beta (C/EBPbeta) (Wang et al., 2008), extracellular signal-regulated kinase 1/2 (ERK 1/2) (Borowiak et al., 2004; Bard-Chapeau et al., 2006; Factor et al., 2010), signal transducer and activator of transcription 3 (STAT3) (Cressman et al., 1995; Li et al., 2002; Moh et al., 2007), and phosphatidylinositol-3-kinase (PI3K)/Akt (Jackson et al., 2008; Haga et al., 2005; Nechemia-Arbely et al., 2011) are representatives. Among these transcription factors and corresponding signaling transductions, the TNF-alpha/NF-KB, IL-6/STAT3, and PI3K/Akt pathways are considered the three major cascades through which platelets exert their effects on liver regeneration (Fig. 2).

The TNF-alpha/NF-KB pathway is activated within 30 minutes of partial hepatectomy and the activation usually lasts no longer than 4-5 hours (Michalopoulos & DeFrances, 1997). NF-KB is found in almost every cell including hepatocytes and non-paranchymal cells. It is a heterodimer composed of two subunits, p65 and p50, which are assembled in the cytosol (Solt & May, 2008). It is inactivated by Inhibitor of NF-KB (IKB) which binds to the p65 subunit. After being stimulated by TNF-alpha, NF-kB is activated by the removal of IkB from the p65 subunit; it migrates to the cell nucleus, where it binds to the promoter of cyclin-D1, which regulates G0/G1-to-S-phase transition (Hinz et al., 1999).

STAT3 is activated more slowly; it becomes detectable 1 to 2 hours after partial hepatectomy and lasts about 4-6 hours (Michalopoulos & DeFrances, 1997). IL-6 binding causes dimerization of the corresponding receptor and the activation of intracellular tyrosine kinase which phosphorylates gp130 and creates a docking site fof STAT3 (Heinrich et al., 1998). STAT3 is phosphorylated and translocates to the nucleus where it promotes the expression of cyclin-D1 and p21 to control the progression of the cell cycle (Turkson & Jove, 2000; Terui et al., 2005). It has been reported that hepatocytic mitosis of STAT3-knockout mice was significantly suppressed after partial hepatectomy in liver regeneration (Haga et al., 2009). The absence of STAT3 in hepatocytes exacerbates liver fibrosis during cholestasis (Shigekawa et al., 2011).

The PI3K/Akt pathway is activated immediately after partial hepatectomy (Murata et al., 2007). The pathway is initiated by the activation of the receptor tyrosine kinases or receptors coupled with G proteins by HGF, IL-6, TNF-alpha, TGF-beta and many other signaling molecules (Osawa et al., 2002; Okano et al., 2003; Tulasne & Foveau, 2008; Kato et al., 2009; Nechemia-Arbely et al., 2011). Met is a tyrosine kinase receptors on the surface of hepatocytes tha binds HGF (Bottaro et al., 1991; Tulasne & Foveau, 2008). HGF/c-met signaling activates PI3K which recruits Akt to the site of membranes, and subsequently phosphorylates Akt (Fresno et al., 2004). Glycogen synthase kinase 3-beta (GSK3-beta) acts downstream of Akt and plays a critical role in liver regeneration by regulating cell growth along with other downstream Akt factors, such as mTOR and 70^{S6K} (Faridi et al., 2003; Latronico et al., 2004; Haga et al., 2005). Phosphorylation of Akt results in activation of GSK3-beta by phosphorylation at serine-9, resulting in accumulation of beta-catenin and cyclin-D1 in the nucleus, which induce DNA synthesis and cellular mitosis of hepatocytes (Gotoh et al., 2003; Chen et al., 2005).

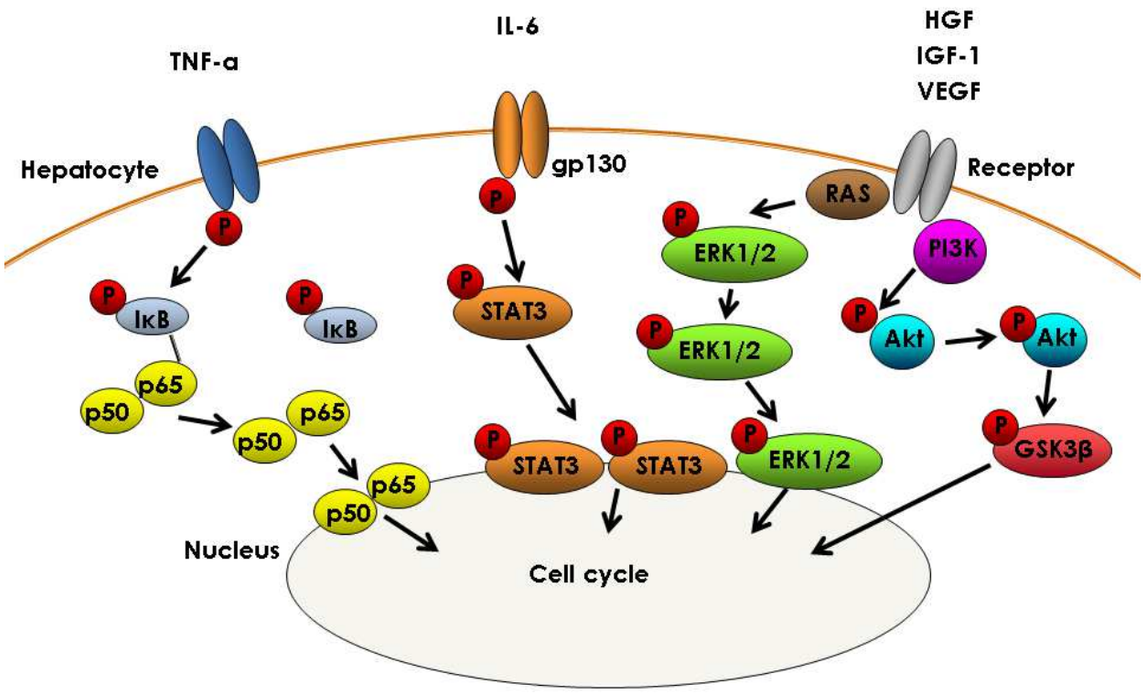


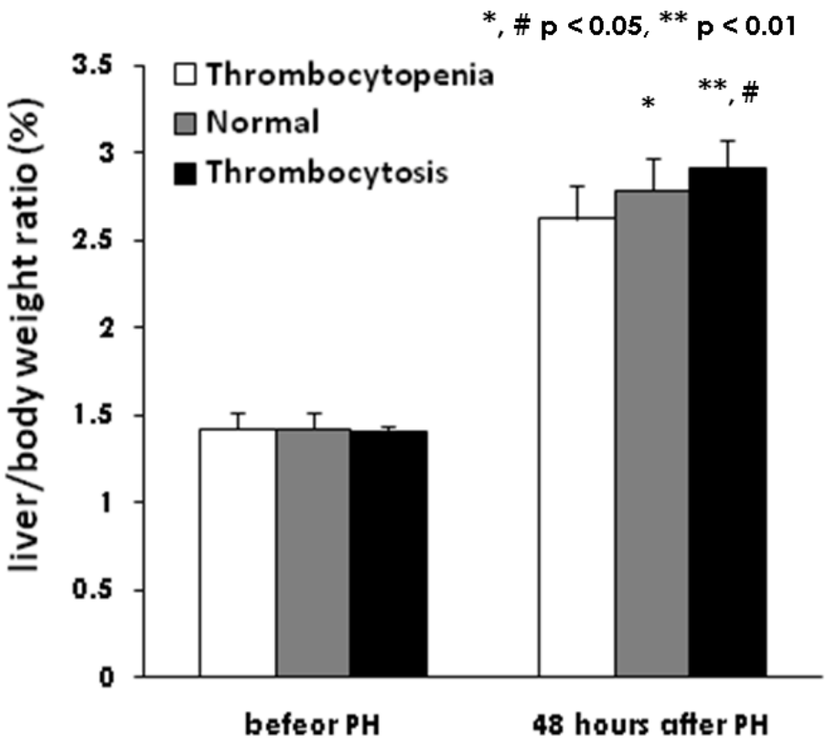
Fig. 2. Cytokines and growth factors for liver regeneration.

3. Effect of platelets on liver regeneration

Our first study was focused on liver regeneration under thrombocytotic conditions induced by thrombopoietin (TPO) (Murata et al., 2007). A 70% partial hepatectomy was carried out and mice were subsequently divided into three groups as follows; untreated mice as normal group, a thrombocytotic group, and a thrombocytopenic group. To induce

thrombocytosis, mice were injected TPO. Anti-mouse platelet monoclonal antibody (Pm-1) was administrated to induce thrombocytopenia. Liver regeneration, cytokine and signaling pathways in the three groups were compared. Differences of platelet accumulation in the liver by using immunohistochemical staining technique were also observed.

The liver/body weight ratios in the thrombocytotic group and normal group were significantly higher than in the thrombocytopenic group, 48 hours after partial hepatectomy. In the thrombocytotic group, the liver/body weight ratio 48 hours after partial hepatectomy was significantly higher than that in normal group (Fig. 3A). The hepatocyte Ki-67 labeling index and hepatocyte mitotic index 48 hours after partial hepatectomy in the thrombocytotic group was obviously higher than that of normal and thrombocytopenic groups (Fig. 3B). Furthermore, the hepatocyte proliferating cell nuclear antigen (PCNA) labeling index 48 hours after partial hepatectomy in the thrombocytopenic group was remarkably lower than that in normal and thrombocytotic groups.



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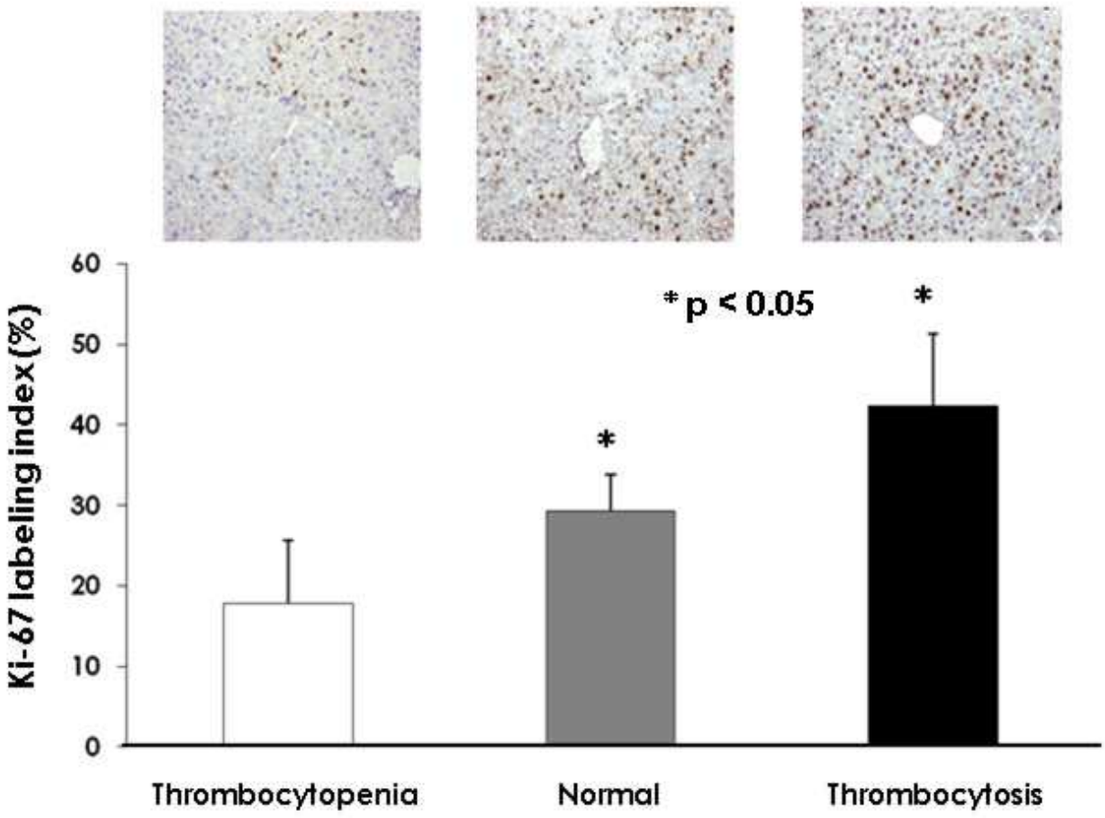


(B)

Fig. 3. Effect of thrombocytosis on liver regeneration after 70% of partial hepatectomy. (A) Liver/body weight ratio before and 48 hours after partial hepatectomy (PH) in thrombocytotic , normal and thrombocytopenic groups. Data were expressed as mean ± SD. *p < 0.05, **p < 0.01 versus thrombocytopenic group. #p < 0.05 normal group versus thrombocytotic group. (B) Ki-67 labeling index 48 hours after partial hepatectomy in thrombocytotic, normal and thrombocytopenic groups. Representative immunohistochemical images are shown. Data were expressed as mean ± SD. *p < 0.05 versus thrombocytopenic group. (Reproduced from Murata et al., 2007, World J Surg with permission.)

HGF and PDGF expression in the liver tissue in thrombocytotic group were significantly higher than in the normal and thrombocytopenic groups. Akt was strongly phosphorylated in the thrombocytotic group compared with the thrombocytopenic group. Activation of Akt in the thrombocytotic group started 5 minutes after partial hepatectomy and persisted for 2 hours. On the other hand, although activation of Akt was seen from 5 minutes after partial hepatectomy in the normal group, activation reduced in 2 hours after partial hepatectomy. In the thrombocytopenic group, Akt was not activated during the first 6 hours after partial hepatectomy. There was no difference in activation in ERK 1/2 and STAT3 among the three groups after partial hepatectomy (Fig. 4).

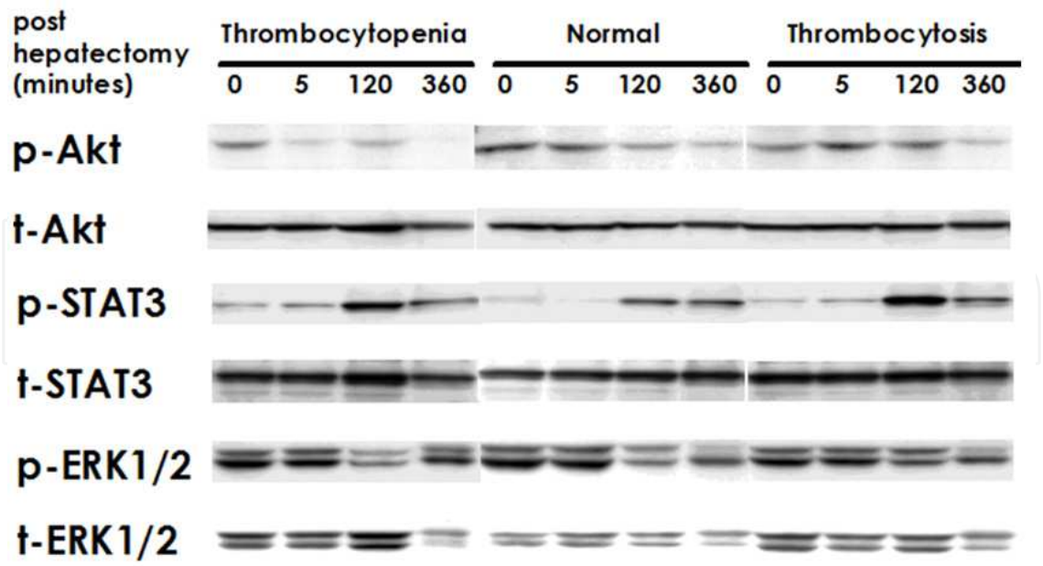


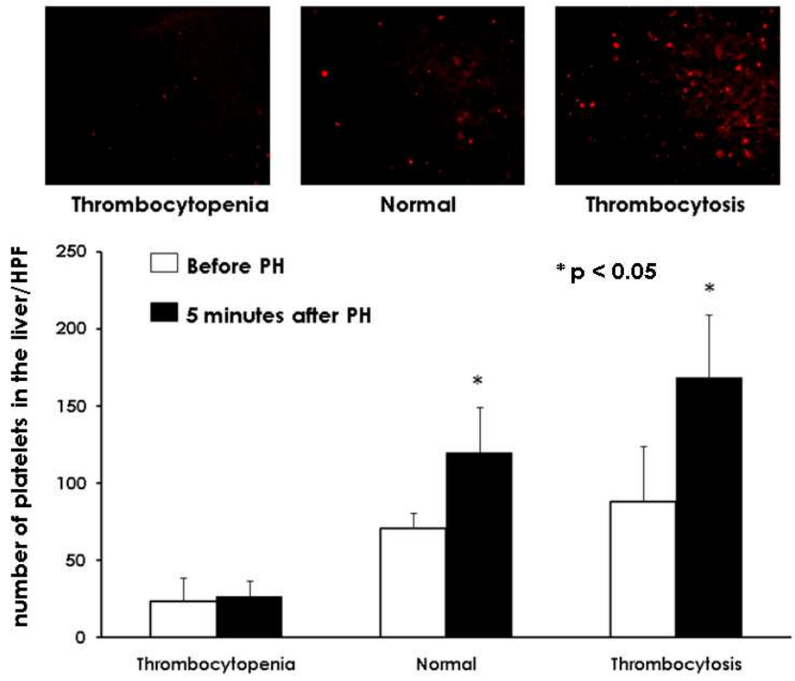
Fig. 4. Effect of platelet increment or reduction on Akt, STAT3, and ERK 1/2 after partial hepatectomy. (Reproduced from Murata et al., 2007, World J Surg. with permission).

Platelet accumulation in the liver was investigated in all groups before and 5 minutes after partial hepatectomy. Platelets accumulated in the residual liver within 5 minutes after partial hepatectomy and a two-fold increase in platelet levels was observed in the normal and thrombocytotic groups (Fig. 5A). On the other hand, in thrombocytotic group, platelets in the residual liver increased remarkably compared with normal and thrombocytopenic groups 5 minutes after partial hepatectomy. However, no increment was observed in thrombocytopenic group. In addition, under transmission electron microscopy, platelets translocated from the liver sinusoidal space into the space of Disse, and they had direct contact with hepatocytes in the thrombocytotic group (Fig. 5B).

These results suggest that platelets accumulate in the liver within a few minutes of partial hepatectomy and cause rapid hepatocyte proliferation through direct contact with hepatocytes, by translocating into the space of Disse.

Taken together, the results described above demonstrate that platelets affect liver regeneration in the acute phase after partial hepatectomy and suggested that the PI3K/Akt pathway is the main signaling pathway involved in platelet mediated liver regeneration.

The following study was done to investigate the role of platelets in liver regeneration using a thrombocytosis model in mice after 90% partial hepatectomy (Myronovych et al. 2008). All mice in the normal group died within 30 hours, predominantly between 20 and 30 hours. In contrast, the survival rate at 30 hours and at one week after partial hepatectomy was 54.5% and 27.3%, respectively in thrombocytotic group (Fig. 6A). Phosphorylation of Akt and STAT3 started earlier and stronger in thrombocytotic group than normal group. Serum albumin levels decreased in both groups after partial hepatectomy, but more rapidly in normal group, and there was a significant difference with higher levels being detected at 24 hours post-hepatectomy in the livers of the thrombocytotic group (Fig 6B). Serum



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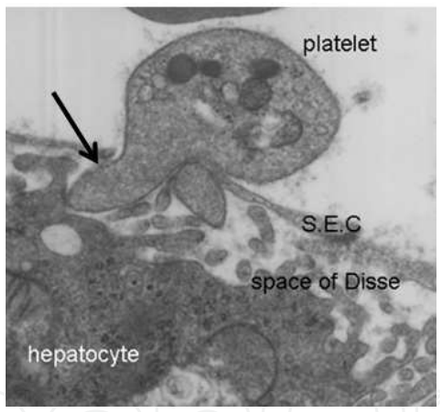


(B)

Fig. 5. Immunohistochemistry and Transmission electron microscopic photograph of the residual liver. (A) Immunohistochemistry of liver frozen section. Red; platelets. Platelets are stained by Pm-1 antibody. Representative images 5 minutes after partial hepatectomy (PH) are shown. Platelets were counted before and 5 minutes after partial hepatectomy in thrombocytopenic, normal, and thrombocytotic groups. Data were expressed as mean ± SD. *p < 0.05 versus before partial hepatectomy. Original magnification X 400. (B) Transmission electron microscopic image of partial hepatectomy in the residual liver 5 minutes after partial hepatectomy in thrombocytotic group. Arrow indicates platelet translocations into the space of Disse through the porosity of a flattened process in a sinusoidal endothelial cell (SEC). Original magnification X 7500. (Reproduced from Murata et al., 2007, World J Surg with permission.)

cholesterol levels were higher in thrombocytotic group at all time points with a significant difference at 24 hours after partial hepatectomy (Fig. 6C). In our measurement of insulin-like growth factor binding protein (IGFBP-1) by real-time PCR, the peak value of IGFBP-1 expression was reached sooner in the thrombocytotic group than in the normal group after partial hepatectomy and decreased moderately afterwards.

The findings described above indicated that liver regeneration occurs even in 90% hepatectomized mice under conditions of thrombocytosis. Platelets contribute to cell cycle progression and metabolic pathways, and maintain liver function after the extended hepatectomy.

We also evaluated the effect of TPO on liver regeneration after partial hepatectomy and on fibrosis under conditions of liver cirrhosis in rats (Murata et al., 2008). Rats were divided into three groups as follows; a normal group without any treatment, a liver cirrhosis (LC) group, and an LC group with a single administration of TPO (LC+TPO). 70% of partial hepatectomy was performed and liver regeneration and anti-fibrotic effects were compared.

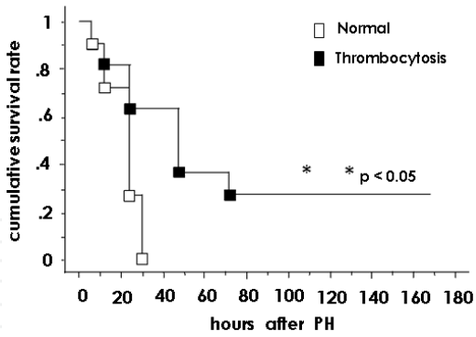
In the LC group, the platelet count in the blood was significantly lower than that in the normal group. In the LC+TPO group, platelet count increased 2-fold higher than that in the normal group (Fig. 7A). The hepatocyte PCNA labeling index 24 hours after partial hepatectomy in the LC group was significantly lower than that in the normal group; the PCNA labeling index in the LC+TPO group was significantly higher than that in the LC group and the same level as that in normal group (Fig. 7B). HGF concentration in liver tissue in the LC+TPO group at the time of partial hepatectomy was clearly higher than that in the normal group. IGF-1 concentration in the liver tissue in LC+TPO group was significantly higher than that in normal group. Fibrotic change around the portal regions in the LC group was more prominent than that in the normal group. In contrast, fibrotic change decreased remarkably in the LC+TPO group (Fig. 7C)

These results described above indicated that a single administration of TPO in cirrhotic liver induces the remarkable increment of the platelets and then improves liver regeneration and fibrosis of cirrhotic liver after 70% of partial hepatectomy.

We examined whether the TPO itself or increased platelets have a hepatocyte-proliferative effect and anti-fibrotic effect on the fibrotic liver. We injected anti-platelet serum (APS) into LC+TPO group (LC+TPO+APS). The platelet count of LC+TPO+APS group decreased remarkably compared with LC and LC+TPO groups (Fig. 8A). PCNA labeling index 24 hours after partial hepatectomy was markedly lower in LC+TPO+APS group than that in LC and LC+TPO groups (Fig. 8B). Furthermore, liver fibrotic area before partial hepatectomy increased significantly in LC+TPO+APS group compared with LC+TPO group (Fig. 8C).

These results clearly indicate that acceleration of liver regeneration and anti-fibrotic effects of TPO administration are induced by increment of platelets, not by TPO itself.

We investigated whether exogenous platelets have the similar encouraging effect on liver regeneration. Platelet-rich plasma (PRP) was infused via the portal vein after 70% partial



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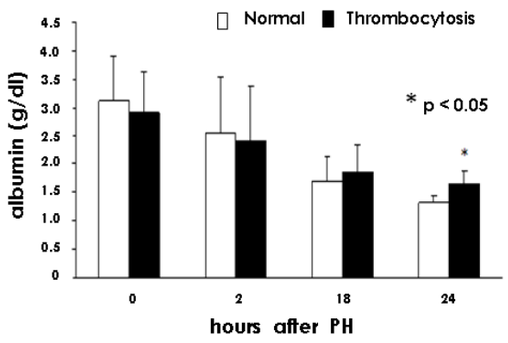

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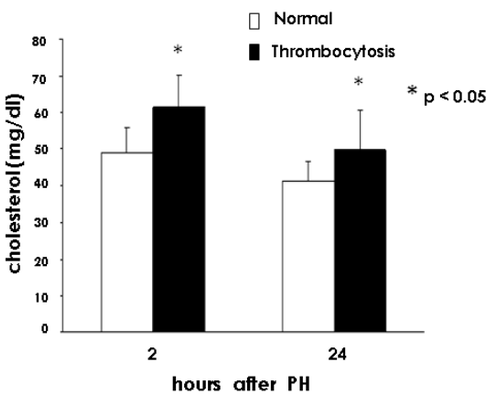
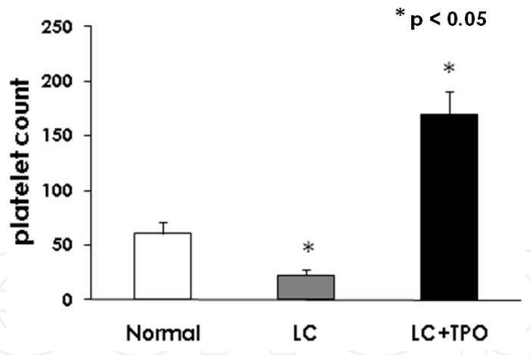


(C)

Fig. 6. Effect of thrombocytosis on survival and metabolic pathways after 90% of partial hepatectomy (PH). (A) Survival rate with Kaplan-Meier method. *p < 0.05 versus normal group. (B) Change in serum albumin concentration. Data were expressed as mean ± SD. *p < 0.05 versus normal group. (C) Change in serum total cholesterol concentration. Data were expressed as mean ± SD. *p < 0.05 versus normal group.
(Reproduced from Myronovych et al., 2008, J Hepatol with permission.)



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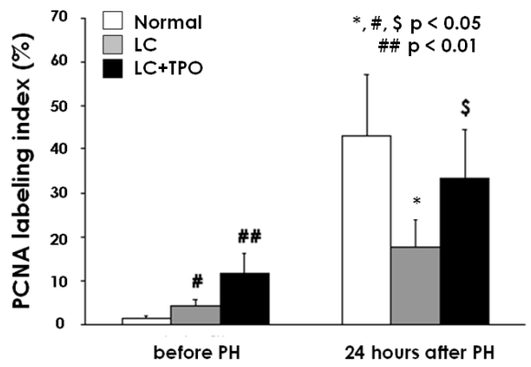

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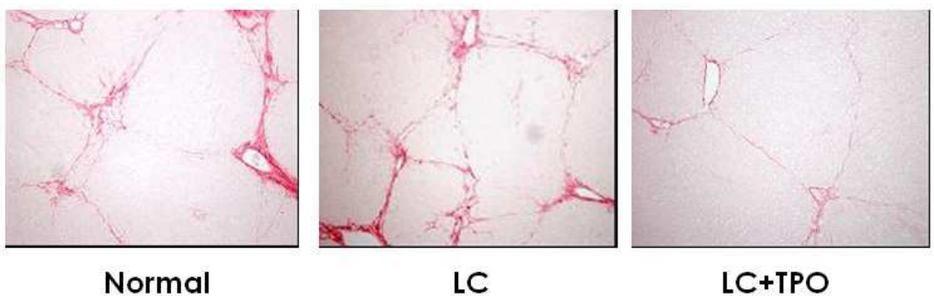


(C)

Fig. 7. Effect of TPO on platelet count, liver regeneration and fibrosis. (A) Platelet count before partial hepatectomy in normal, LC, and LC+TPO groups. Data were expressed as mean ± SD. *p < 0.05 versus normal group. (B) The hepatocyte PCNA labeling index before and 24 hours after partial hepatectomy (PH) in normal, LC, and LC+TPO groups. Data were expressed as mean ± SD. #p < 0.05, ##p < 0.01 versus normal group before partial hepatectomy. *p < 0.05 versus normal group 24 hours after partial hepatectomy. $p < 0.05 versus LC group 24 hours after partial hepatectomy.
(C) Fibrotic change in the liver in normal, LC, and LC+TPO groups. Representative image in each group. Sirius red staining of liver sections. Original magnification × 200. (Reproduced from Murata et al., 2008, Ann Surg with permission.)

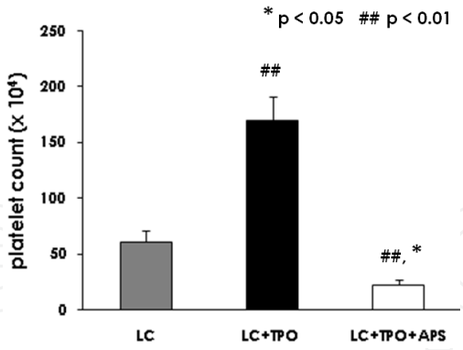
hepatectomy and residual liver regeneration was evaluated in rats (Matsuo et al., 2011). To clarify the mechanisms by which platelet promote liver regeneration, we also analyzed the dynamics of platelets infused in the liver before and after partial hepatectomy using an intravital microscope (IVM).

The liver/body weight ratio 24 hours after partial hepatectomy was significantly higher in PRP transfused group (PRP+) than in the normal saline administered group (PRP-). The hepatocyte Ki-67 labeling index was significantly higher in the PRP+ group than that in the PRP- group. Akt and ERK 1/2 became phosphorylated earlier in the PRP+ group than in the PRP- group, whereas phosphorylation of STAT3 did not apparently differ between the two groups. Under IVM, although platelets flowed fast and few of them rolled and adhered in the liver sinusoids before partial hepatectomy, a significant proportion of platelets accumulated in the liver sinusoids and flowed slowly with adhesion and rolling after partial hepatectomy. The findings in this experiment indicate that exogenous platelets also promote liver regeneration.

Since the anatomy of porcine liver is similar to that of the human, and porcine liver is useful for mimicking human liver surgery, we evaluated the effect of platelets in anti-liver damage and liver regeneration using pigs (Hisakura et al., 2010). To induce thrombocytosis, pigs received TPO administration (TPO+) or were performed splenectomy (Sp+). Pigs underwent 80% partial hepatectomy and were assigned to either TPO-, TPO+, Sp-, or Sp+ groups; liver damage, histological findings including necrotic changes, ballooning, cholestasis, and liver regeneration were compared among these groups. Serum aspartate aminotransferase levels in the TPO+ group were significantly lower than that in the TPO- group on day 2 after partial hepatectomy. Serum alanine aminotransferase levels in the Sp+ group were significantly lower than that in the Sp- group on day 2 after partial hepatectomy. Serum alkaline phosphatase levels in the TPO+ and the Sp+ groups were significantly lower than those in the TPO- and the Sp- group at 6 hours and on day 2 after partial hepatectomy. Histological analysis for cholestasis, ballooning, and hepatocyte necrosis was carried out by using a scoring system (Table. 1). Although cholestasis, ballooning, and hepatocyte necrosis were observed in zone 2 in TPO- and Sp- groups, structure was mostly preserved in TPO+ and Sp+ group (Fig. 9). On the other hand, the liver/body weight ratio and the hepatocyte PCNA labeling index showed no significant difference among the groups on day 2 and 7 after partial hepatectomy.

Under transmission microscopy, the sinusoidal endothelial linings were destroyed and detached into sinusoidal space with the enlargement of the spaces of Disse and the cytoplasm of sinusoidal endothelial cells was swollen with secondary lysosomes 2 hours after partial hepatectomy in TPO- or Sp- group (Fig. 10). In contrast, the structure of the endothelial lining was well preserved in TPO+ and Sp+ group.

Although there was no direct evidence of platelets in promoting liver regeneration in this experiment, the results indicated that increase in the number of platelets protect sinusoidal linings from disturbance and prevent acute liver damage after extended hepatectomy.



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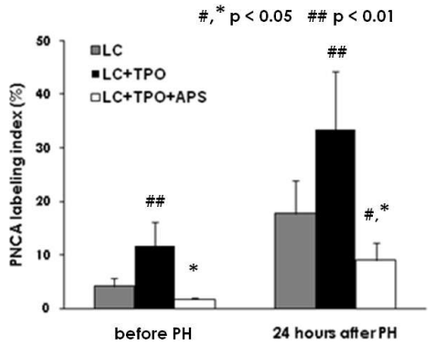

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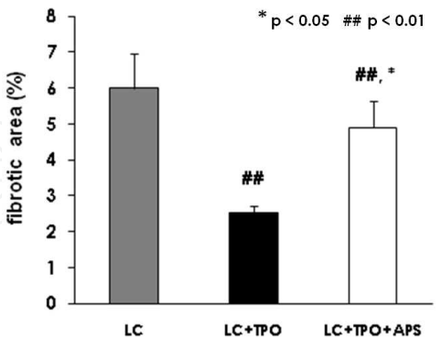


(C)

Fig. 8. Effect of platelets on liver regeneration and fibrosis of the liver. (A) Platelet count before partial hepatectomy (PH). (B) PCNA labeling index before and 24 hours after partial hepatectomy. (C) Fibrotic area of the liver in LC, LC+TPO, and LC+TPO+APS groups. Data were expressed as mean ± SD. #p < 0.05, ##p < 0.01 versus LC group. *p < 0.05 versus LC+TPO group. (Reproduced from Murata et al., 2008, Ann Surg with permission.)

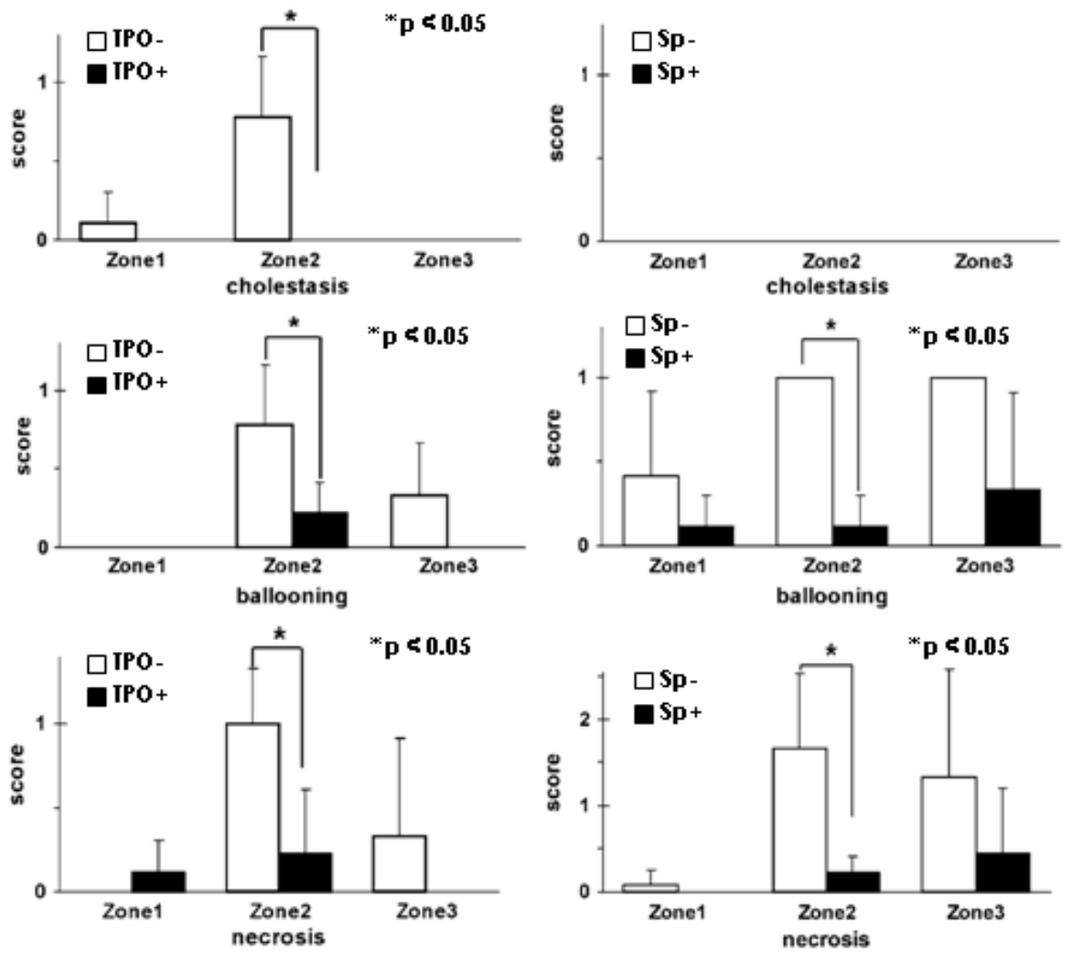


Fig. 9. Semiquantitative scoring for cholestasis, ballooning, and hepatocyte necrosis in TPO-, TPO+, Sp-, and Sp+ groups. Data are expressed as means ± SD. *p < 0.05 versus TPO- group or Sp- group. (Reproduced from Hisakura et al, 2010, J Hepatobiliary Pancreat Sci with permission.)

Feature	Scoring system	
cholestasis	0	No
	1	Yes
Ballooning	0	No
	1	Yes
Necrosis	0	None
	1	Small foci
	2	Confluent areas
	3	Bridging necrosis

Table 1. Scoring system of cholestasis, ballooning, and hepatocyte necrosis. (Reproduced from Hisakura et al., 2010, *J Hepatobiliary Pancreat Sci* with permission.)

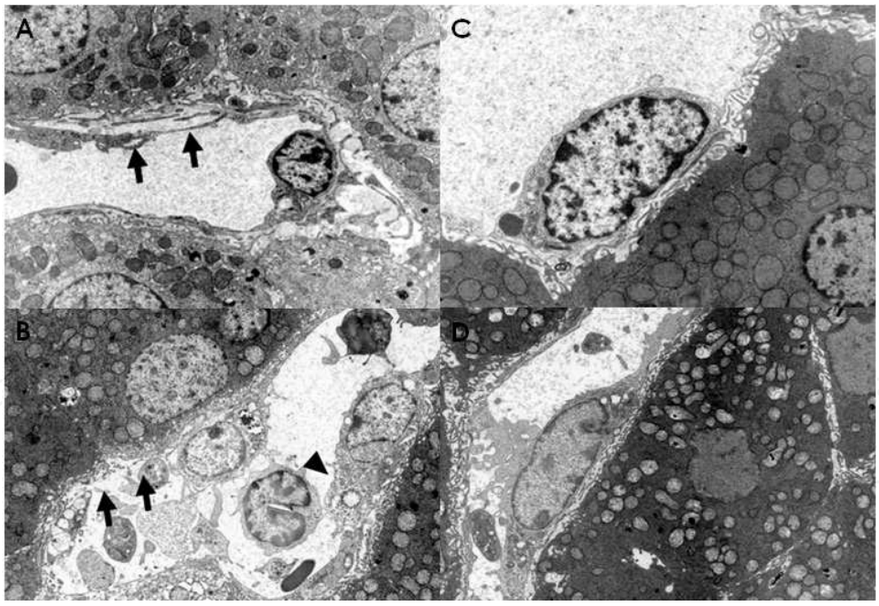


Fig. 10. Transmission electron microscopic findings 2 hours after partial hepatectomy. Magnification × 6000. In TPO- (A), and Sp- groups (B), the sinusoidal endothelial lining was destroyed and detached into the sinusoidal space with enlargement of the space of Disse (arrows), and the cytoplasm of sinusoidal endothelial cells were swollen with secondary lysosomes (arrow head). In contrast, in TPO+ (C), and Sp+ groups (D), the sinusoidal endothelial cells were well preserved. (Reproduced from Hisakura et al., 2010, *J Hepatobiliary Pancreat Sci* with permission.)

4. Mechanisms of direct effect of platelets on liver regeneration

Up to the beginning of the 21st century, there was no report regarding the effect of platelets on liver regeneration. Two studies were reported in which platelets promoted liver regeneration (Murata et al., 2004; Lesurtel et al., 2006). We reported that platelets accumulate in the liver and translocate actively to the space of Disse through fenestrae of LSECs after partial hepatectomy, which enables platelets to contact directly with hepatocytes (Murata et al., 2007). To clarify the role of the direct contact of platelets with hepatocytes, we investigated by using co-culturing chamber systems where platelets and hepatocytes were separated by a permeable membrane (Matsuo et al., 2008). To elucidate characteristics of the direct contact, four groups of separated co-culture were prepared as follows: a without platelet group (platelet-), a mixed co-culture group (co-mix), a separated co-culture group (co-sep), a group with mixed cells (the upper mix group: upper-mix), and the thrombin-stimulated group (thrombin stimulated) were prepared (Fig. 11). TLR2 cells, the murine immortalized primary hepatocytes, in the lower chamber were counted 72 hours after incubation. In the upper-mix group, platelets induced significant proliferation of hepatocytes in the lower chamber, whereas the proliferation in co-sep group was almost the

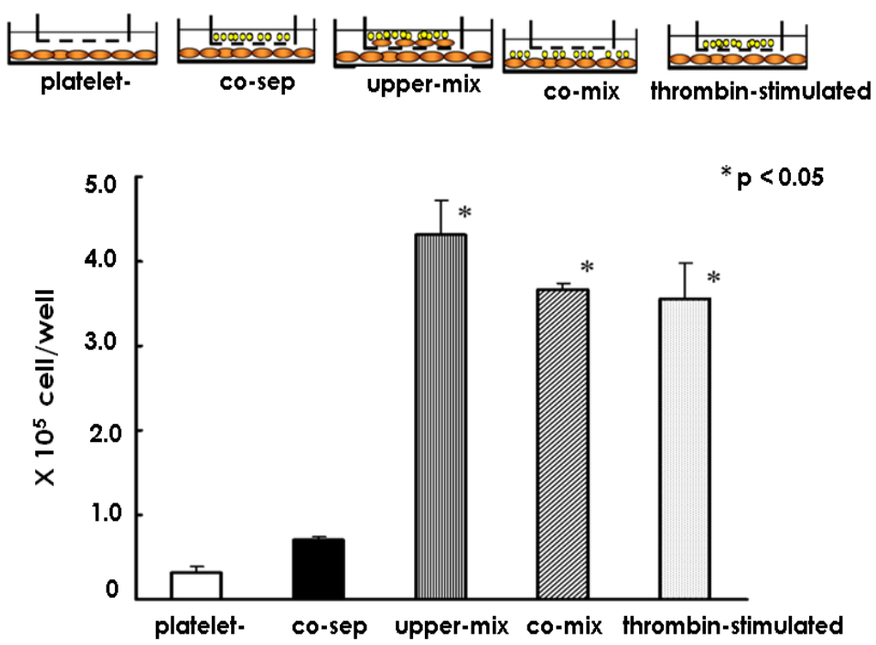


Fig. 11. Co-culture system to elucidate the characteristics of direct contact.
Without platelet group (platelet-): neither hepatocytes nor platelets were seeded in the upper chamber. Separated co-cultured group (co-sep): platelets were seeded in the upper chamber. Upper mix group (upper-mix): hepatocytes were seeded in the upper chamber and overlayered with platelets. Co-mixed group (co-mix): platelets and hepatocytes were seeded in the lower chamber. Thrombin stimulated group (thrombin-stimulated): platelets in the upper chamber were stimulated with thrombin to release soluble factors such as cytokines and growth factors. Hepatocytes in the lower chamber are counted after 72 hours of incubation. Data are expressed as means SD. *$p < 0.05$ versus platelet-.
(Reproduced from Matsuo et al., *J Surg Res* with permission.)

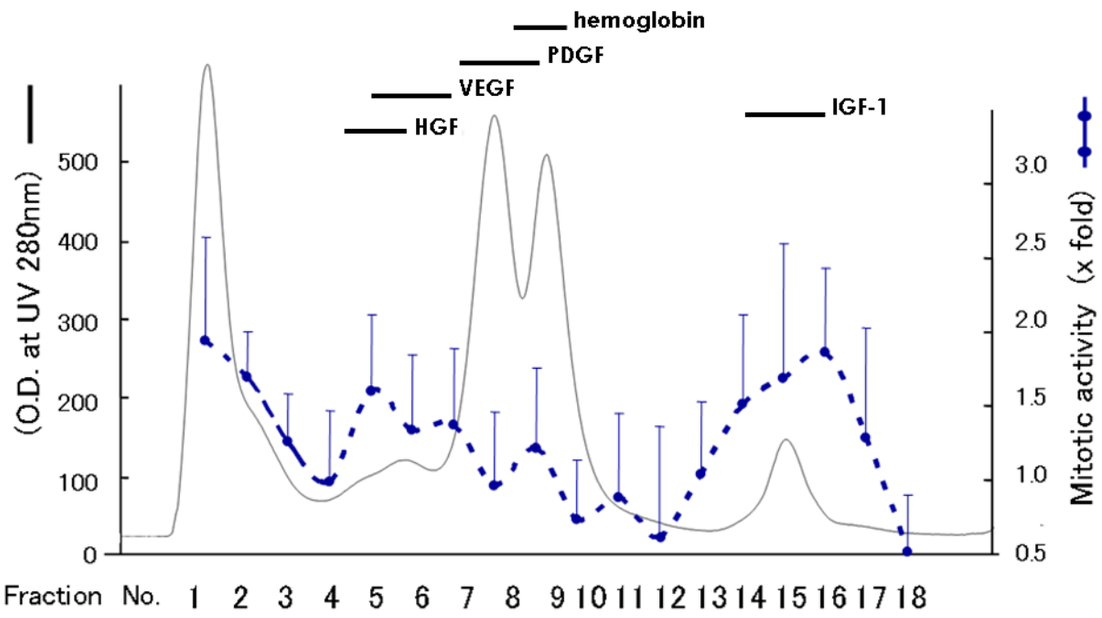
same level as that of platelet- group. Moreover, this effect in the upper-mix group was of the same level as in the co-mix group. These results indicate that, upon direct contact with hepatocytes, platelets released soluble factors that induce hepatocyte proliferation. A proliferative effect was also observed in the thrombin-stimulated group, despite there being no direct contact between platelets and hepatocytes (Fig. 11).

To clarify which component of platelets had an effect on hepatocyte proliferation, the mitogenic activity of the whole disrupted platelets, the soluble fraction, and the membrane fraction were evaluated. The whole disrupted platelets and the soluble fraction had significant proliferative effects, whereas the membrane fraction did not have the effect. To determine which element of the platelet soluble factor exerted the proliferative effect, platelet extracts were gel-excluded into 18 fractions, and mitogenic activity of each fraction was evaluated on BrdU assay. Mitogenic activity was strongly induced in the fraction of HGF, VEGF, and IGF-1 (Fig. 12A). In addition, when hepatocyte signals were analyzed in response to growth factors, HGF, IGF-1, and VEGF strongly activated the Akt and the ERK1/2 pathways, whereas PDGF and serotonin did not induced activation (Fig. 12B). For further confirmation of the platelets soluble factors, IGF-1 and HGF inhabitation using anti-IGF-1 and anti-HGF antibodies significantly inhibited hepatocyte proliferation.

The results of this examination indicated that the direct contact between platelets and hepatocytes triggered the release of soluble factors from the platelets such as IGF-1 and HGF, which caused a proliferative effect on the hepatocytes.

We assessed the direct effect of platelets using Kupffer cell depletion model (Murata et al., 2008). Liposome-encapsulated dichloromethylene diphosphonate (Cl2-MDP) was used for the depletion of Kupffer cells. Mice were divided into four groups as follows: mice without any treatment (normal), mice with Kupffer cell depletion (KD), mice with thrombocytosis caused by injection of thrombopoietin (thrombocytosis), and mice undergoing Kupffer cell depletion and thrombocytosis by injection of thrombopoietin (TKD). Each group of mice underwent 70% partial hepatectomy, and liver regeneration, cytokine and growth factors expression, and phosphorylation of Akt were assayed in the groups.

The liver/body weight ratio in KD group was significantly lower than that in normal group 48 hours after partial hepatectomy. The liver/body weight ratio in TKD group was almost the same as that in normal group. In thrombocytotic group, the liver/body weight ratio was significantly higher than that in normal group (Fig. 13 A). The hepatocyte mitotic index of the KD group 48 hours after partial hepatectomy was significantly lower than that in normal group. And, the hepatocyte mitotic index in the TKD group was almost the same as that in normal group. Furthermore, the hepatocyte mitotic index in thrombocytotic group was significantly higher than those in other groups. Moreover, the hepatocyte PCNA labeling index 48 hours after partial hepatectomy in the KD group was significantly lower compared with normal group. And, in the TKD group, it was significantly higher than that in KD group and the same as that in thrombocytotic and normal groups (Fig. 13 B).



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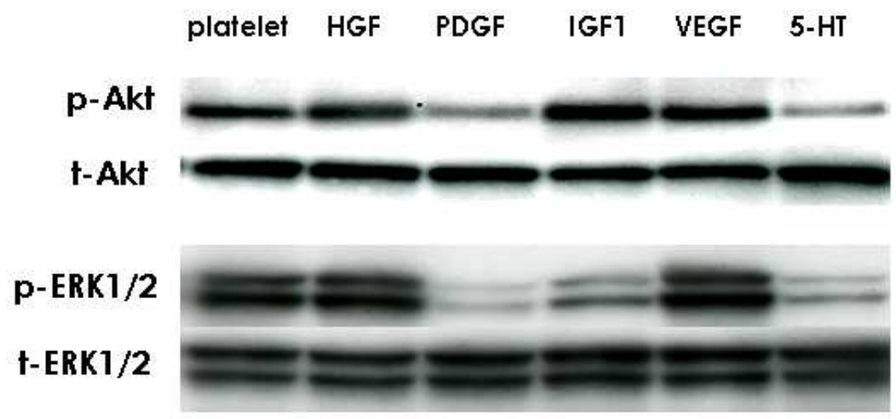
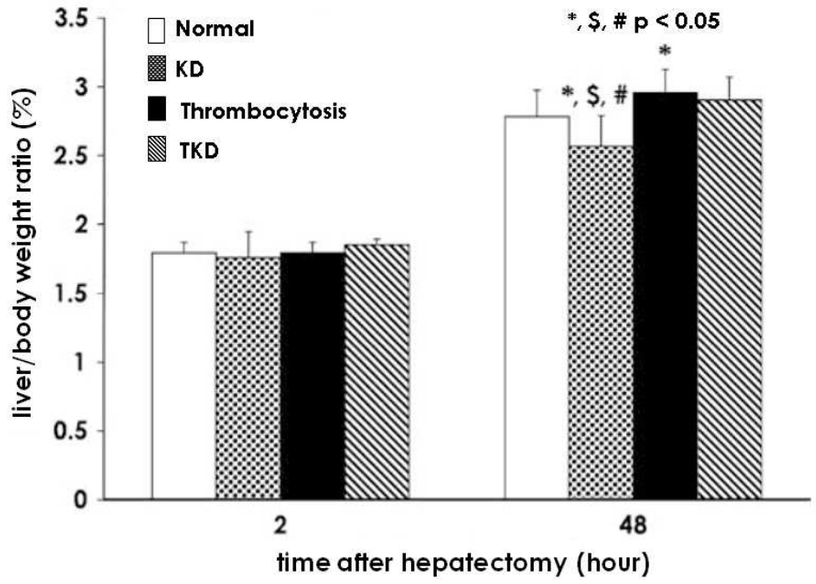


(B)

Fig. 12. Gel exclusion chromatography of platelet extracts and mitotic activities.
(A) The platelet extracts were gel filtrated on Superdex G200 gel. The solid line shows the resulting absorbance profile at 280 nm. The broken line shows the mitogenic activity of each fraction. Fraction 1 and 2 were nonspecifically macroaggregated proteins. Significant mitogenic activity was observed in fraction 1 ,2, 5-7, and 14-17. On western blotting, fractions 4-6 were rich in HGF, fraction 5-7 were rich in VEGF, fractions 7-9 were rich in PDGF, and fraction 14-17 were rich in IGF-1. Data were expressed as means ± SD of each experiments. (B) Cellular signals of hepatocytes stimulated by platelets and PDGF, HGF, PDGF, IGF-1, VEGF, and Serotonin (5-HT). (Reproduced from Matsuo et al., 2008, J Surg Res with permission.)



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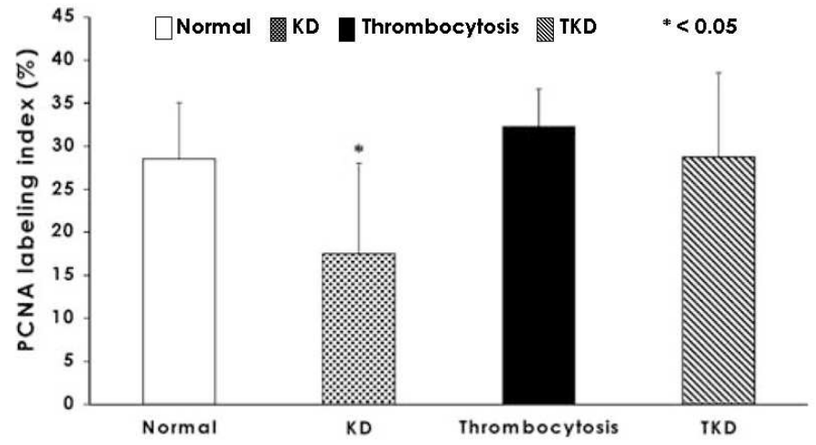


(B)

Fig. 13. Liver regeneration indexes under Kupffer cell depletion and thrombocytosis. (A) Liver/body weight ratio 2 and 48 hours after partial hepatectomy. Data are expressed as means ± SD. *p < 0.05 versus normal group, $p < 0.05 versus thrombocytosis; #p < 0.05 versus TKD group. (B) The hepatocyte PCNA labeling index 48 hours after partial hepatectomy. Data are expressed as means ± SD. *p < 0.05 versus normal group. (Reproduced from Murata et al., 2008, World J Surg with permission.)

The liver content of TNF-alpha, HGF, and IGF-1 was assessed in normal, KD, and TKD groups. TNF-alpha expression increased and reached the peak 2 hours after partial

hepatectomy in normal group, whereas it remained low in KD and TKD groups. HGF concentration in the liver tissue in TKD group at the time of partial hepatectomy was significantly higher than that in normal group, and it persisted 6 hours after partial hepatectomy. At the same time, IGF-1 concentration in the liver tissue in KD and TKD groups at the time of partial hepatectomy was significantly lower than that in normal group, and IGF-1 concentration in the TKD groups was higher than that in the KD group. Furthermore, Akt was strongly phosphorylated in normal group compared with the KD group. In the TKD group, phosphorylation of Akt was started at the time of PH and lasted until 120 minutes after PH, and it was almost the same level as it was in normal group.

Platelet accumulation 2 hours after partial hepatectomy was investigated in each group. Platelet accumulation in the KD group demonstrated a significant decrease compared with the normal group. Moreover, platelet accumulation in the TKD group showed a significantly higher level than that in the KD group, and it was almost the same level as that in the normal group. In the thrombocytotic group, platelet accumulation increased significantly compared with the normal group. Transmission electron microscopy demonstrated that in the thrombocytotic group, platelets translocated from the liver sinusoidal space to the space of Disse and were in direct contact with hepatocytes at 5 minutes after hepatectomy.

These results clearly demonstrate that platelets promote liver regeneration under conditions of Kupffer cell depletion. Increase of platelets recruited platelets in the liver tissue and elevated and HGF concentrations in the liver, which activated downstream signaling transduction and hepatocyte mitosis.

In conclusion, our previous studies clarified the direct effect of platelets in promoting liver regeneration. The mechanism is explained as follows; after partial hepatectomy, platelets accumulate in the liver, they translocate to the space of Disse and release growth factors such as IGF-1 and HGF through direct contact with hepatocytes. The growth factors stimulate initiation of hepatocyte mitosis, which eventually promote liver regeneration. Especially in human, since it was reported that human platelets do not contain a significant amount of HGF (Nakamura et al., 1989), IGF-1 is the most important mediator for liver regeneration, which is contained in human platelets (Fig. 14).

5. The effect with liver sinusoidal endothelial cells

LSECs comprise 70% of the sinusoidal cells (Knook & Sleyster, 1976; Smedsrod et al., 1990). By construction of a thin and continuous layer, the sinusoidal endothelium forms the structural barrier, separating the hepatic parenchyma from blood constituents passing through the liver. Unlike other vascular endothelial cells, LSECs have large cytoplasmic gaps without basal membranes. These enable maximal contact between circulating blood and hepatocytes to help exchange various soluble macromolecules and nano-particles such as lipoproteins and endocytosis (Braet & Wisse, 2002). LSECs are involved in liver regeneration as well as Kupffer cells and hepatic stellate cells, and they are known to produce immunoregulatory and pro-inflammatory cytokines including HGF, interleukin-1 (IL-1), IL-6, and interferon. In addition, they synthesize eicosanoids, particularly TXA_2, prostaglandin E_2, as well as synthesizing important regulators of vascular tone, such

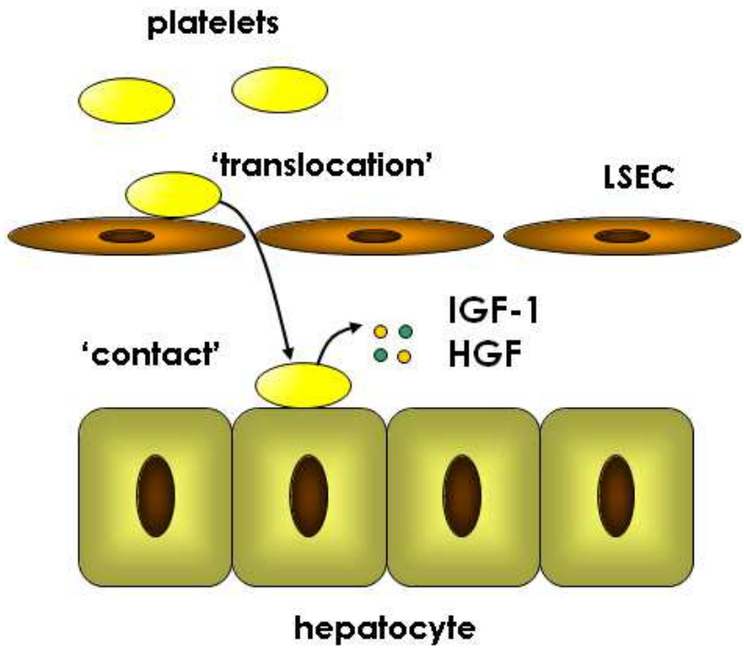


Fig. 14. Scheme for liver regeneration promoted directly by platelets.
Platelets translocate to the space of Disse and release growth factors such as IGF-1 and HGF through direct contact with hepatocytes. The growth factors subsequently induce initiation of mitosis.

as nitric oxide and endothelin (Wisse et al., 1996; Vollmar & Menger, 2009; Ping et al., 2006). IL-6 produced by LSECs and Kupffer cells is one of the important components of early signaling pathways in liver regeneration, and it activates the acute phase of protein synthesis by hepatocytes as part of the overall inflammatory response (Gauldie et al., 1992; Michalopoulos & DeFrances, 1997). After hepatectomy, plasma IL-6 concentration is reported to increase from 6 hours to a peak by 24 hours (Rai et al., 1996; Badia et al., 1998). IL-6 binds to its receptor on hepatocytes, which subsequently leads to phosphorylation of STAT3 monomers (Fausto et al., 2006). STAT3 homodimerizes and translocates to the nucleus, where it stimulates transcription of a number target genes such as cyclin-D1 and p21.

The relationship between platelets and LSECs has been well-documented in ischemia/reperfusion injury models. Rolling and adhering of leukocytes on LSECs with subsequent interaction with platelets is the important pathogenesis of ischemia/reperfusion injury (Montalvo-Jave et al., 2008; Croner et al., 2006, Pak et al., 2010). It was also reported that transient interaction, i.e., rolling, and permanent adhesion of platelets to the post-ischemic hepatic endothelium stimulate platelet activation and expression of endothelial adhesion molecules (Massberg et al., 1998; Khandoga et al., 2003). There have not, however, been any prior studies focused on the relationship between human platelets and LSECs with regards to liver regeneration.

To clarify the role of platelets in liver regeneration in relation to LSECs, we used co-culturing chamber systems where platelets and LSECs could be separated by a permeable

membrane (Kawasaki et al., 2010). We used TMNK-1 cells (immortalized human LSECs), instead of primary LSECs, since their utility and efficiency was confirmed in the previous basic research (Matsumura et al., 2004).

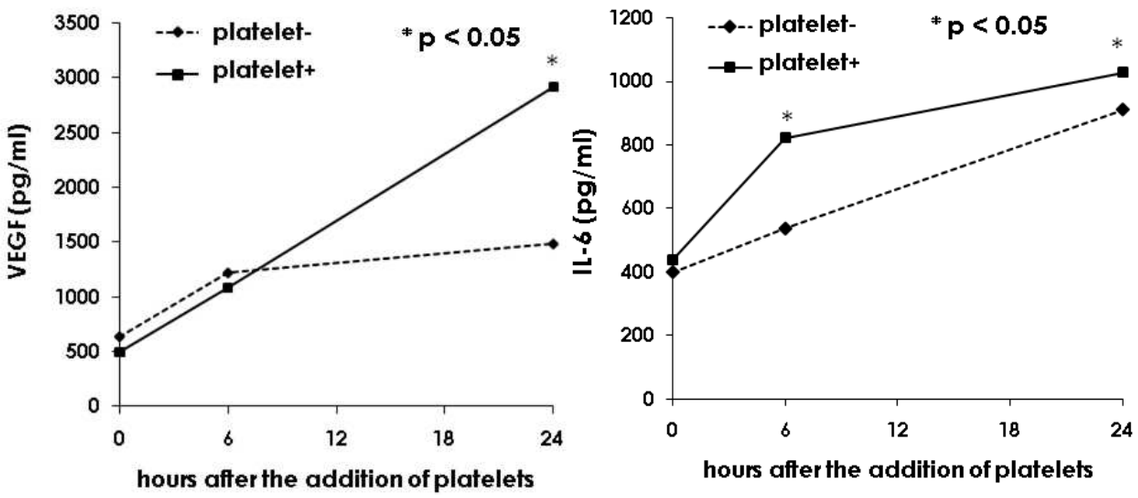
Proliferation of LSECs and concentrations of IL-6 and VEGF in the supernatant were significantly higher in the group in which LSECs were co-cultured with human platelets (platelet+ or platelet+ mixed) than they were in the group in which LSECs were cultured without human platelets (platelet-) (Fig. 15A,B). However, when the platelets and LSECs were cultured separately (platelet+separated), no significant increase of IL-6 was observed (Fig. 15B). These results indicated that human platelets increase proliferation of LSECs and induced IL-6 release from LSECs and that the direct contact between platelets and LSECs is required for the production of IL-6. BrdU uptake of the primary hepatocytes in the group administered with the supernatant co-cultured with platelets and LSECs was significantly higher than that in the group administered with the supernatant cultured without platelets. When a specific antagonist for sphingosine 1-phosphate (S1P) 2 receptors were added to LSECs and co-cultured with platelets, the concentration of IL-6 showed significant decrease (Fig. 16A). On the contrary, the concentration of IL-6 was clearly increased in the group administered with S1P compared with those without S1P (Fig. 16B). These results revealed that S1P in platelets played important roles in liver regeneration by release of IL-6 from LSECs.

S1P is generally expressed in human plasma. It belongs to the class of lipid mediators and has been shown to regulate diverse biological processes, including proliferation, survival migration, or cytoskeletal reorganization (Yatomi et al., 2000; Xia & Wadham., 2011). S1P is produced from platelets and interacts with endothelial cells under the conditions of critical platelet-endothelial interactions, i.e., thrombosis, angiogenesis, and atherosclerosis (Yatomi et al., 2000). It was reported that the biological effect of S1P is partially mediated by endothelial nitric oxide synthetic activation and subsequent nitric oxide formation; extracellular S1P could contribute to sinusoidal protection and remodeling in alcoholic liver injury (Zheng et al., 2006). However, it was also described that S1P in human hepatic myofibroblast has an anti-mitogenic effect by increasing expression of TGF-β (Ikeda et al., 2003). As described above, S1P has various kinds of biophysical effects.

From the results of our experiment, the promotive effect of platelets on liver regeneration could be explained by follows; the direct contact between platelets and LSECs induce S1P release from platelets, which subsequently induce excretion of IL-6 from LSECs. LSEC-derived IL-6 promotes DNA synthesis of hepatocytes through STAT3 pathway (Fig. 17).

6. The role of Kupffer cells on liver regeneration

Kupffer cells are the principal constituents of the non-paranchymal cells of the liver (Malik et al., 2002). They locate within the lumen of the liver sinusoids, and are adherent to the LSECs. Kupffer cells play a role as macrophages against bacteria, bacterial endotoxins and microbial debris derived from gastrointestinal tract (Bilzer at al., 2006). Kupffer cells have been postulated to play a key role in liver regeneration after partial hepatectomy, and they could produce important biologically-active mediators that have both stimulatory and inhibitory influence on hepatocyte proliferation after hepatectomy (Boulton et al., 1998). Except for a report stating augmentation of the early phase of liver regeneration with Kupffer cell depletion (Meijer et al., 2000), depletion of Kupffer cells is basically well-known



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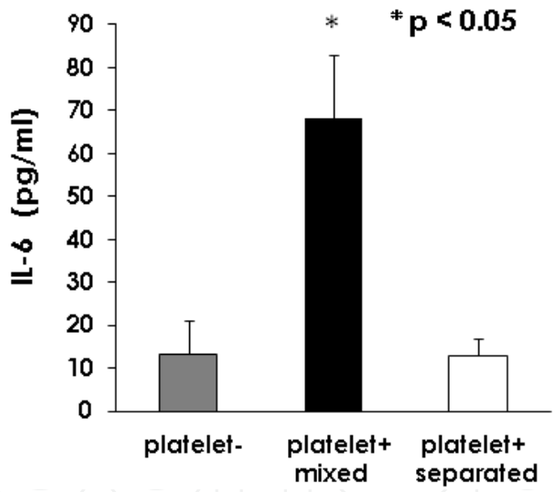
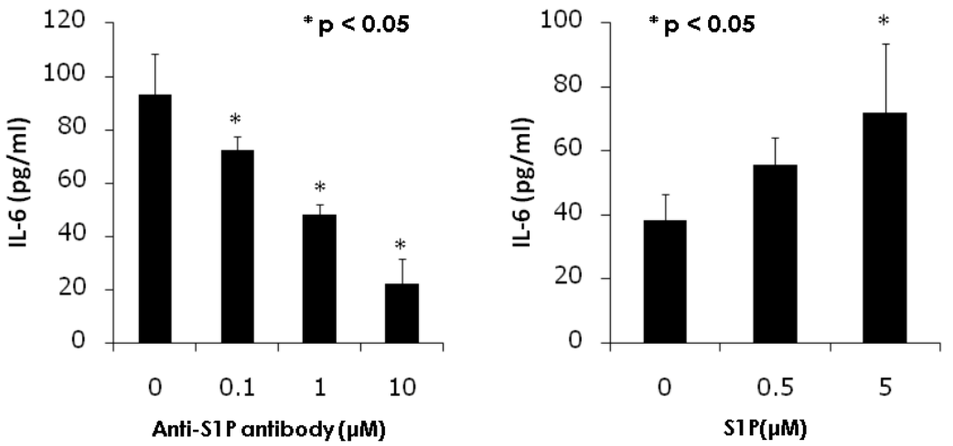


(B)

Fig. 15. Assay of IL-6 and VEGF in the supernatant of cultured LSECs after the addition of platelets, and the necessity of contact with platelets for excretion of IL-6 from LSECs. (A) The amounts of IL-6 and VEGF in the supernatant of LSECs were measured 0, 6, and 24 hours after the addition of platelets. Data are expressed as means ± SD. *p < 0.05 versus platelet– group. (B) To investigate the necessity of direct contact between platelets and LSECs, LSECs were cultured for 6 hours with platelets mixed (platelet+mixed) or platelets separated (platelet + separated), and the excretion of IL-6 from LSECs was measured. Data are expressed as means ± SD. *p < 0.05 versus platelet+separated group. (Reproduced from Kawasaki et al., 2010, *J Hepatol* with permission.)



(A) (B)

Fig. 16. Effects of inhibitor of S1P and S1P on excretion of IL-6 from LSECs
(A) Excretion of IL-6 from LSECs was evaluated using a specific antagonist for S1P2 receptors. LSECs were cultured with platelets for 6 hours, and the amount of IL-6 in the supernatant of LSECs was measured. Data are expressed as means ± SD. *p < 0.05 versus inhibitor- group. (B) To determine whether S1P had an effect on excretion of IL-6 from LSECs, LSECs were cultured with S1P for 6 hours, and the amount of IL-6 in the supernatant of TMNK-1 cells was measured. Data are expressed as means ± SD. *p < 0.05 versus S1P- group. (Reproduced from Kawasaki et al., 2010, *J Hepatol* with permission.)

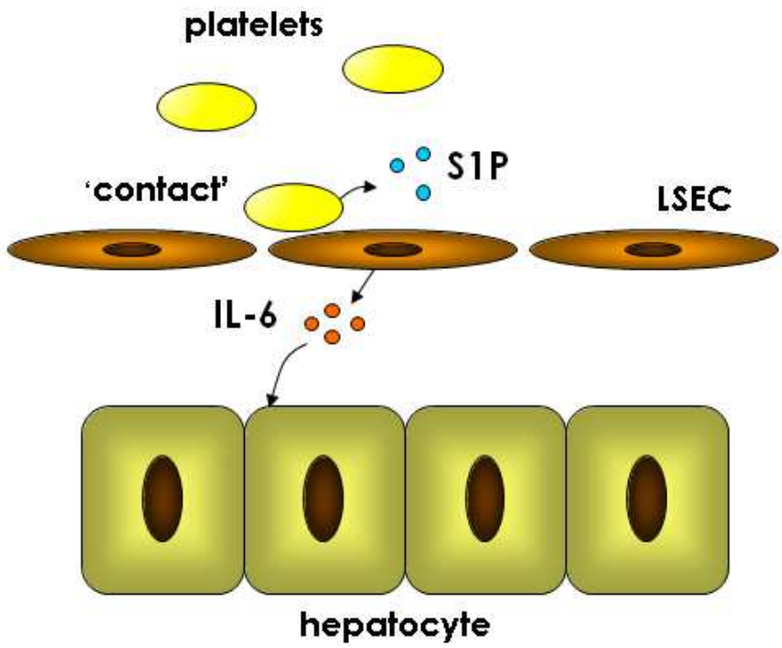


Fig. 17. Scheme for liver regeneration promoted by LSECs and platelets.
The direct contact between platelets and LSECs triggers excretion of S1P from platelets, which subsequently causes excretion of IL-6 from LSECs. IL-6 from LSECs promotes DNA synthesis of hepatocytes.

to exert an inhibitory influence on liver regeneration by alteration of hepatic cytokine expression (Takeishi et al, 1999). A critical early event following partial hepatectomy is the increase in plasma levels of TNF-alpha. In support of this view, an experiment using antibody against TNF-alpha has demonstrated significant reduction of hepatocyte proliferation (Akerman et al., 1992). Mice lacking TNF receptor-1 were shown to demonstrate severe impairment in liver regeneration (Yamada et al., 1998). Activation of the TNF receptor increases hepatic level of the NF-KB in both hepatocytes and non-paranchymal cells, and it is followed by production and release of IL-6 from Kupffer cells. Kupffer cells are assumed to be one of the most important sources of both TNF-alpha and IL-6 (Kahn et al., 1994; Decker., 1998). This is supported by the report that Kupffer cell-depleted mice failed to increase TNF-alpha, and IL-6 levels were equivalent to the level of Kupffer cell-competent mice after partial hepatectomy (Abshagen et al., 2007).

The relationship between platelet and Kupffer cells has been also well-documented in ischemia/reperfusion injury models. Platelets act in concert with the activated Kupffer cells and leukocytes, and a triangular interaction between these cells has been demonstrated as the main mechanism of the injury (Vollmar & Menger, 2009; Sindram et al., 2001). It was reported that when rats with depletion of Kupffer cells were subjected to ischemia and reperfusion, platelet adhesion in sinusoids was suppressed and, as consequence, attenuation of sinusoidal perfusion failure and endothelial damage were seen (Nakano et al., 2008). It is also reported that Kupffer cells produce PAF, which is a potent phospholipid mediator of platelet aggregation (Karidis et al., 2006). PAF is also believed to play important roles in the acute liver injury with ischemia/reperfusion (Karidis et al., 2006; Toledo-Pereyra & Suzuki, 1994), liver graft dysfunction (Hashikura et al., 1994), and post-operative liver failure after extended hepatectomy (Mizuno et al., 2001). As shown above, the role of platelets in relation to Kupffer cells have been described mainly with inflammatory injuries of the liver.

Nakamura et al. described a different character of Kupffer cell function associated with platelets. They reported that in response to LPS, IL-1, and TNF-alpha, platelets accumulated in the liver and a large number of platelets were found in the space of Disse (Endo et al., 1992, 1993; Nakamura et al., 1998). They also observed that platelets in the liver sinusoids were mostly surrounded by well-developed cell processes of Kupffer cells without being phagocytosed (Nakamura et al., 1998). However, depletion of Kupffer cells resulted in abolition of hepatic accumulation and migration of platelets (Nakamura et al., 1998). Although the precise mechanism was not clear, these reports indicated that cellular interaction between platelets and Kupffer cells plays an important role in platelet behavior in the liver.

Previously, we reported that even under condition of Kupffer cell depletion, platelets accumulated in the liver in the thrombocytotic state and promoted liver regeneration by direct contact with hepatocytes through their migration from the liver sinusoidal space to the space of Disse (Murata et al., 2008). In our recent study using SCID mice with human platelet transfusion, we demonstrated that concentrations of mouse-derived TNF-alpha and IL-6 in the liver tissue after 70% of partial hepatectomy were significantly higher in the mice with platelet transfusion than in the mice without transfusion. These results may indicate that platelet transfusion enhances secretory activity of Kupffer cells after hepatectomy. Furthermore, in the mice with platelet transfusion, significant accumulation and activation of platelets transfused

were observed in the liver after hepatectomy. Although a few platelets transfused were adhering to the Kupffer cells in the mice without hepatectomy, the majority of platelets adhered to the surface of Kupffer cells in the mice with hepatectomy. It is insufficient to conclude only from these findings, however, it was assumed that platelets promote liver regeneration by interactions with Kupffer cells after hepatectomy. In other words, after hepatectomy, Kupffer cells induce accumulation and activation of platelets in the liver by direct adhering, and function of Kupffer cells are enhanced by the accumulated platelets. As described above, liver regeneration is promoted by the direct effect of growth factors released from platelets and by the paracrine effect of Kupffer cells enhanced by platelets (Fig. 18).

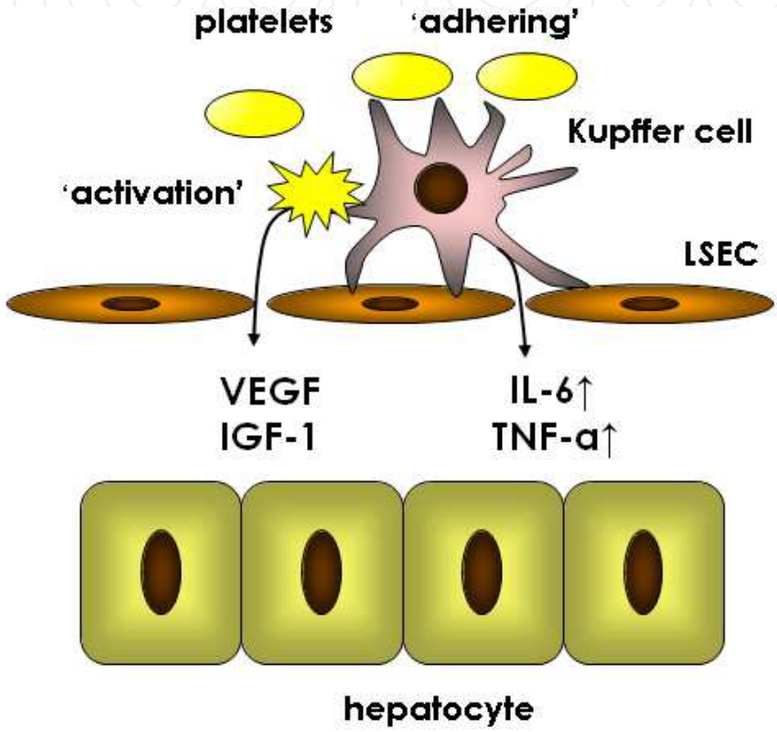


Fig. 18. Scheme for liver regeneration promoted by Kupffer cells

After partial hepatectomy, the activated Kupffer cells induce accumulation and activation of platelets through direct adhering. By the direct effect of growth factors released from platelets, and by the paracrine effect of Kupffer cells enhanced by platelets, liver regeneration is promoted.

7. Conclusion

In this chapter we have described our previous reports of platelets in promoting liver regeneration and the three different mechanisms by which platelete promote liver regeneration, i.e., 1) the direct effect on hepatocytes, 2) the cooperative effect with LSECs, and 3) the collaborative effect with Kupffer cells. Platelets are blood components that contain various kinds of biologically-active growth factors and cytokines. Nowadays artificial platelets (Bode & Fischer, 2007; Okamura et al., 2009), TPO formulae (Rhodes & Stasi, 2010), and freeze-dried platelets (Hoshi et al., 2007) are being developed and are

beginning to be utilized in clinical settings; the importance and effects of platelets will become more apparent in the near future. With several lines of evidence showing platelets to be effective in anti-fibrosis (Watanabe et al., 2008; Kodama et al., 2010), anti-apoptosis (Hisakura et al., 2011), and liver regeneration, platelet therapy would open a new avenue to develop novel strategies for the treatments of liver diseases. Through these researches, we believe that platelet therapy could offer a therapeutic strategy for liver regeneration after extended hepatectomy, liver injuries or small grafts in liver transplantation.

8. Acknowledgement

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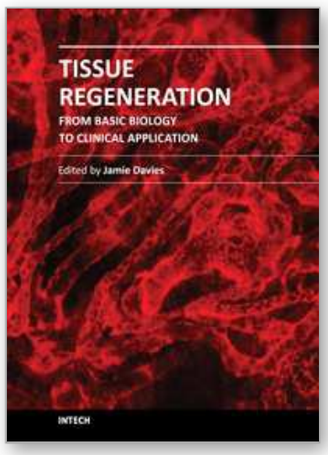

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When most types of human tissue are damaged, they repair themselves by forming a scar - a mechanically strong 'patch' that restores structural integrity to the tissue without restoring physiological function. Much better, for a patient, would be like-for-like replacement of damaged tissue with something functionally equivalent: there is currently an intense international research effort focused on this goal. This timely book addresses key topics in tissue regeneration in a sequence of linked chapters, each written by world experts; understanding normal healing; sources of, and methods of using, stem cells; construction and use of scaffolds; and modelling and assessment of regeneration. The book is intended for an audience consisting of advanced students, and research and medical professionals.

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