Research Article

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Activation of human liver sinusoidal endothelial cell by human platelets induces hepatocyte proliferation

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Background & Aims: We previously reported that platelets promote hepatocyte proliferation. In this study, we focused on the role of platelets in liver sinusoidal endothelial cells (LSECs) in addition to their role in hepatocyte in liver regeneration.

Methods: Immortalized human LSECs (TMNK-1) were used. The LSECs were co-cultured with human platelets, and the proliferation of LSECs and the excretion of growth factors and interleukin-6 (IL-6) were subsequently measured. The main factor from platelets which induced the excretion of IL-6 from LSECs was determined using inhibitors of each component contained in the platelets. The need for direct contact between platelets and LSECs was investigated using cell culture inserts. The proliferation of human primary hepatocytes was measured after the addition of the supernatant of LSECs cultured with or without platelets.

Results: The number of LSECs cocultured with platelets significantly increased. Excretion of IL-6 and vascular endothelial growth factor (VEGF) increased in LSECs with platelets. JTE-013, a specific antagonist for sphingosine 1-phosphate (S1P) 2 receptors, inhibited the excretion of IL-6 from LSECs after the addition of platelets. When the platelets and LSECs were separated by the cell culture insert, the excretion of IL-6 from LSECs was decreased. DNA synthesis was significantly increased in human primary hepatocytes cultured with the supernatant of LSECs with platelets.

Conclusions: Platelets promote LSEC proliferation and induce IL-6 and VEGF production. Direct contact between the platelets and LSECs and S1P, that are contained in platelets, were involved in the excretion of IL-6 from LSECs. IL-6 from LSECs induced proliferation of parenchymal hepatocytes.

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Introduction

Liver regeneration is a critical issue related to clinical morbidity and mortality after an extended hepatectomy or other types of liver injury [1,2]. The precise mechanism of liver regeneration is still unclear [3]. Hepatocytes have a mitogenic response to various growth factors and cytokines such as hepatocyte growth factor (HGF), interleukin-6 (IL-6), tumor necrosis factor alpha, epidermal growth factor (EGF), tissue growth factor beta, and platelet-derived growth factor (PDGF) [4]. These factors stimulate their own receptors in the cell membrane and activate transcription factors and signal transduction pathways; i.e., nuclear factor kappa-B, signal transducer and activator of transcription 3 (STAT3), extracellular signal-regulated protein kinase (ERK) 1/2, protein kinase B (Akt), and activator protein-1. Subsequently, hepatocyte proliferation occurs [5,6]. Kupffer cells, liver sinusoidal endothelial cells (LSECs), and hepatic stellate cells are involved in the mechanism of liver regeneration [7]. LSECs have been reported to produce HGF, IL-6, and nitric oxide (NO), and to promote hepatocyte proliferation after hepatectomy [8].

Platelets contain many growth factors, such as PDGF, VEGF, EGF, and insulin-like growth factor 1 (IGF-1) in the alpha granules [9]. It was reported that platelets contribute to bone regeneration [10] and dermal wound healing [11]. Platelets also have an important role in liver regeneration [12]. However, few studies have investigated the relationship between platelets and LSECs. Recently, some studies have focused on the role of platelets in hepatic ischemia/reperfusion (I/R) [13]. Activated platelets have been considered to be involved in hepatic injury and contribute to the development of apoptosis in LSECs after I/R [14,15]. In our previous studies, we revealed that platelets stimulate hepatocyte proliferation in *in vitro* and *in vivo* models [13,16–23]. These results suggest that growth factors contained in the platelets are responsible for the activation of the Akt, ERK1/2, and STAT3 pathways. In this study, we focused on sphingosine 1-phosphate (S1P) in addition to growth factors. S1P is a lipid mediator contained in platelets and was reported to regulate a broad variety of cellular processes such as cell proliferation, apoptosis, calcium homeostasis, vascular maturation, or angiogenesis [24].

Keywords: Liver regeneration; Liver non-parenchymal cell; Growth factor; Interleukin-6; Sphingosine 1-phosphate.

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Abbreviations: LSECs, liver sinusoidal endothelial cells; IL-6, interleukin-6; VEGF, vascular endothelial growth factor; S1P, sphingosine 1-phosphate; HGF, hepatocyte growth factor; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; STAT3, signal transducer and activator of transcription 3; ERK1/2, extracellular signal-regulated protein kinase 1/2; NO, nitric oxide; IGF-1, insulin-like growth factor 1; I/R, ischemia/reperfusion; PRP, Platelet-rich plasma; ACD, acid citrate dextrose; HSA, human serum albumin; ANOVA, analysis of variance.

No previous studies have mentioned the relationship between platelets, human LSECs, and hepatocytes in liver regeneration. The purpose of the present study was to clarify the role of platelets in the stimulation of LSECs and the effect of activated LSECs by platelets on hepatocyte proliferation.

Materials and methods

Preparation of platelets

Blood was obtained from healthy volunteers after obtaining their signed informed consent. Platelet-rich plasma (PRP) was obtained by centrifuging anticoagulated blood containing acid citrate dextrose (ACD) in a 1:4 volume ratio at 200*g* at 4 °C for 10 min. The platelets were washed twice by centrifugation of PRP at 1000*g* at 4 °C for 15 min and resuspended in citrate buffer (120 mM NaCl, 4.26 mM NaH_2PO_4, 5.5 mM glucose, 4.77 mM sodium citrate, and 2.35 mM citric acid) adjusted to pH 6.5. Finally, the platelets were suspended in 0.5% human serum albumin (Wako Pure Chemical Industries, Tokyo, Japan)–PBS (HSA–PBS).

Liver sinusoidal endothelial cell line (TMNK-1)

TMNK-1 is a human liver sinusoidal endothelial cell line established by the transfection of SV40T and hTERT that proliferates *in vitro* [25]. TMNK-1 cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C in DMEM medium (Wako) supplemented with 10% FBS (Hyclone, Road Logan, UT) and 1% penicillin and streptomycin (Invitrogen, Grand Island, NY).

Isolation and culture of human hepatocytes

Human adult normal liver samples were obtained during partial hepatic resection performed at the University of Tsukuba Hospital. These patients were seronegative for hepatitis B and C with normal liver function and none had received chemotherapy just before the operation. All sampled specimens of liver tissues were pathologically normal. All samples were collected with the informed consent of the patients. Furthermore, analysis of the samples was approved by the Ethics Committee and the institutional review board of University of Tsukuba Hospital. Hepatocytes were prepared using the Seglen collagenase perfusion technique [26]. The isolated parenchymal cell suspension had viability between 90% and 95% on the trypan blue exclusion test. The hepatocytes were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C in Williams' E medium (Sigma, St. Louis, MO) supplemented with 200 μM L-glutamine (Invitrogen), 10% FBS (Hyclone), 100 M dexamethasone (Sigma), 1 g/ml insulin (Sigma), 1% penicillin, and streptomycin (Invitrogen).

Effects of platelets on TMNK-1 proliferation

The effect of platelets on TMNK-1 proliferation was evaluated by counting TMNK-1 cells cocultured with platelets. Briefly, the TMNK-1 cells (3×10^4 in 1 ml DMEM medium supplemented with 10% FBS) were seeded in 6-well plates. After incubation at 37 °C for 24 h, the medium was changed to the serum-free DMEM medium (1 ml/well) and cultured for 2 h. Then, platelets suspended in 0.5% HSA–PBS (100 μl/well) at different densities (1×10^7/ml, and 1×10^8/ml) were added to each well. After incubation at 37 °C for 24 and 48 h, the TMNK-1 cells were counted.

Western blot analysis of transcription factor in TMNK-1

To investigate the mechanism of platelet-induced TMNK-1 proliferation, we analyzed the TMNK-1 signals that were activated by platelets. TMNK-1 cells were pre-cultured under confluent conditions in 6-well culture plates, and the medium was changed to serum-free DMEM medium (2 ml/well) and cultured for 2 h. Then, platelets suspended in 0.5% HSA–PBS at 1×10^9/ml density were added to each well (200 μl/well). TMNK-1 cells were harvested at 30, 60, 90, 120, and 180 min after the addition of platelets. For Western blot analysis, TMNK-1 lysates were subjected to 10% SDS–PAGE and transferred to a PVDF membrane. The following antibodies were used as primary antibodies: STAT3 (9132), phospho-STAT3 (9131), Akt (9272), phospho-Akt (9271), ERK1/2 (9102), phospho-ERK1/2 (9101), GSK3β (9315), and phospho-GSK3β (9336) (Cell Signaling, Beverly, MA).

Assay of HGF, IL-6, VEGF, and IGF-1 in the supernatant of cultured TMNK-1

TMNK-1 cells were pre-cultured under confluent conditions in 6-well culture plates, and the medium was changed to the serum-free medium (2 ml/well) and cultured for 2 h. Then, platelets suspended in 0.5% HSA–PBS at 1×10^9/ml density were added to each well of the group cocultured with platelets (200 μl/well). Nothing was added to each well of the group cultured without platelets. After incubation at 37 °C for 0, 6, and 24 h, the supernatant of TMNK-1 was sampled and centrifuged at 1000*g* for 15 min. Then the amounts of HGF, IL-6, VEGF, and IGF-1 in the supernatant were measured. In the present study, to quantify the amounts of IL-6, VEGF, and IGF-1, commercially available Quantikine ELISA kits (R&D Systems, Minneapolis, MN) and the Human specific HGF ELISA kit (Institute of Immunology, Tokyo, Japan), were used.

Effects of inhibitors on excretion of IL-6 from TMNK-1 with platelets

To determine which platelet component had an effect on the excretion of IL-6 from TMNK-1, we inhibited the excreted soluble factors; i.e., VEGF and PDGF; using neutralizing antibodies or receptor antibodies and evaluated the excretion of IL-6 from TMNK-1. The following antibodies were diluted in each defined medium and used: Anti-human VEGF Antibody MAB293 (R&D Systems), Anti-human IGF-I Antibody AF-291-NA (R&D Systems), PDGFR Tyrosine Kinase Inhibitor IV (Calbiochem, San Diego, CA), and a specific antagonist for S1P2 receptors JTE-013 (Cayman Chemical, Ann Arbor, MI). TMNK-1 cells were pre-cultured under confluent conditions in 24-well culture plates, and the medium was changed to the serum-free medium (1 ml/well). Then, various concentrations of the receptor antibodies PDGFR Tyrosine Kinase Inhibitor IV and JTE-013 were added to the cells and cultured for 2 h. The platelets were then suspended in serum-free medium at a 1×10^9/ml density and were added to each well (100 μl/well). For assays involving the neutralizing antibodies MAB293 and AF-291-NA, the platelets were pre-incubated with the antibodies for 30 min at 37 °C before being added to TMNK-1. After incubation at 37 °C for 6 h, the supernatant of TMNK-1 was sampled and centrifuged at 1000*g* for 15 min. Then amounts of IL-6 in the supernatant were measured using Quantikine ELISA kits.

Effects of S1P on excretion of IL-6 from TMNK-1

To determine whether S1P had an effect on the excretion of IL-6 from TMNK-1, we added S1P to TMNK-1 and evaluated the excretion of IL-6 from TMNK-1. TMNK-1 cells were pre-cultured under confluent conditions in 24-well culture plates, and the medium was changed to the serum-free medium (1 ml/well) and cultured for 2 h. Then, various concentrations of S1P (Sigma) were added to the cells. After incubation at 37 °C for 6 h, the supernatant of TMNK-1 was sampled and centrifuged at 1000*g* for 15 min. Then amounts of IL-6 in the supernatant were measured using Quantikine ELISA kits.

Necessity of contact with platelets for excretion of IL-6 from TMNK-1

To investigate the necessity of direct contact between platelets and TMNK-1, TMNK-1 cells and platelets were cocultured using a cell culture insert (pore size; 0.4 μm; Becton–Dickinson, Bedford, MA), and excretion of IL-6 from TMNK-1 was measured as described above. TMNK-1 cells were pre-cultured under confluent conditions in 6-well culture plates. The medium was then changed to the serum-free medium (2 ml/well) and cell culture inserts were placed in each well, and cultured for 2 h. Then, platelets suspended in serum-free medium at 1×10^9/ml density were added to the lower chamber of each well of the group cocultured with platelets (mixed), or to the upper chamber of each well of the group cocultured with platelets (separated) (200 μl/well). Only serum-free medium was added to each well of the group cultured without platelets (200 μl/well). After incubation at 37 °C for 6 h, the supernatant of TMNK-1 was sampled and centrifuged at 1000*g* for 15 min. Then, the amounts of IL-6 in the supernatant were measured using Quantikine ELISA kits.

Effects of the supernatants of TMNK-1 on hepatocyte proliferation

The effect of the supernatant of TMNK-1 on hepatocyte proliferation was evaluated using the BrdU assay kit (Roche Diagnostics GmbH, Penzberg, Germany). The supernatant of TMNK-1 cultured with or without platelets was sampled as mentioned in assay of HGF, IL-6, VEGF, and IGF-1 in the supernatant of cultured TMNK-1 (cultured for 24 h). Primary hepatocytes (3×10^3 in 100 μl Williams' E medium supplemented with 10% FBS) were seeded in 96-well plates. After incubation at 37 °C for 24 h, the medium was changed to the serum-free Williams' E

medium (100 μl/well) and cultured for 2 h. Then, the supernatant of TMNK-1 was added to each well (100 μl/well). After incubation at 37 °C for 24 and 48 h, BrdU uptake was measured according to the manufacture's instruction.

Western blot analysis of the transcription factor in primary hepatocytes

The supernatant of TMNK-1 cultured with platelets was sampled as mentioned in assay of HGF, IL-6, VEGF, and IGF-1 in the supernatant of cultured TMNK-1 (cultured for 24 h). Primary hepatocytes were pre-cultured in 6-well culture plates, and the medium was changed to the serum-free Williams' E medium (2 ml/well) and cultured for 2 h. Then, the supernatant of TMNK-1 was added to each well (105 μl/well). Hepatocytes were harvested at 5, 10, 15, 30, and 60 min after the addition of the supernatant. For Western blot analysis, hepatocyte lysates were subjected to 10% SDS–PAGE and transferred to a PVDF membrane. The following antibodies were used as primary antibodies: STAT3, phospho-STAT3, Akt, phospho-Akt, ERK1/2, GSK3β, phospho-GSK3β, and phospho-ERK1/2.

Effects of IL-6 in the supernatants of TMNK-1 on hepatocyte proliferation

To investigate the effect of IL-6 in the supernatant of TMNK-1 on hepatocyte proliferation, we inhibited the effect of IL-6 using neutralizing antibody MAB206 (R&D Systems) and BrdU uptake was measured. The supernatant of TMNK-1 cocultured with platelets was pre-incubated with or without MAB206 for 30 min at 37 °C before being added to hepatocytes. We used cryopreserved hepatocytes (viability 83%) and all other methods were the same as described in effects of the supernatants of TMNK-1 on hepatocyte proliferation. The final concentration of MAB206 was 10 μg/ml.

Statistical analysis

All data are expressed as means ± standard deviation of samples. Statistical analyses were carried out with the Mann–Whitney *U*-test and one-way analysis of variance (ANOVA), and significant data were examined by the Bonferroni–Dunn multiple comparisons post hoc test. In all cases, a p value <0.05 was considered significant.

Results

Effects of platelets on TMNK-1 proliferation

Proliferation of TMNK-1 in the group co-cultured with platelets (1×10^7/ml) was significantly higher than in the group cultured without platelets after 48 h incubation (Fig. 1A).

Signal transduction of TMNK-1

The phosphorylation of Akt, GSK3β, and ERK1/2 was analyzed in TMNK-1 cells stimulated by platelets for up to 180 min (Fig. 1B). The Akt and ERK1/2 pathways were activated within 30 min after the addition of platelets; the STAT3 pathway was not activated (data not shown).

Assay of HGF, IL-6, VEGF, and IGF-1 in the supernatant of cultured TMNK-1

Concentrations of IL-6 and VEGF were significantly increased in the supernatant of the group cocultured with platelets compared to the supernatant of the group cultured without platelets at 6 and 24 h (IL-6) or 24 h (VEGF) after the addition of platelets (Fig. 2). HGF was not detected in either supernatant of groups cultured with or without platelets (data not shown).

Effects of inhibitors on excretion of IL-6 from TMNK-1 with platelets

Concentration of IL-6 was significantly decreased in the group administered with JTE-013 compared to those without JTE-013 after the addition of platelets and antibodies. There was no significant difference in the concentration of IL-6 in groups with and without administration of other antibodies (Fig. 3).

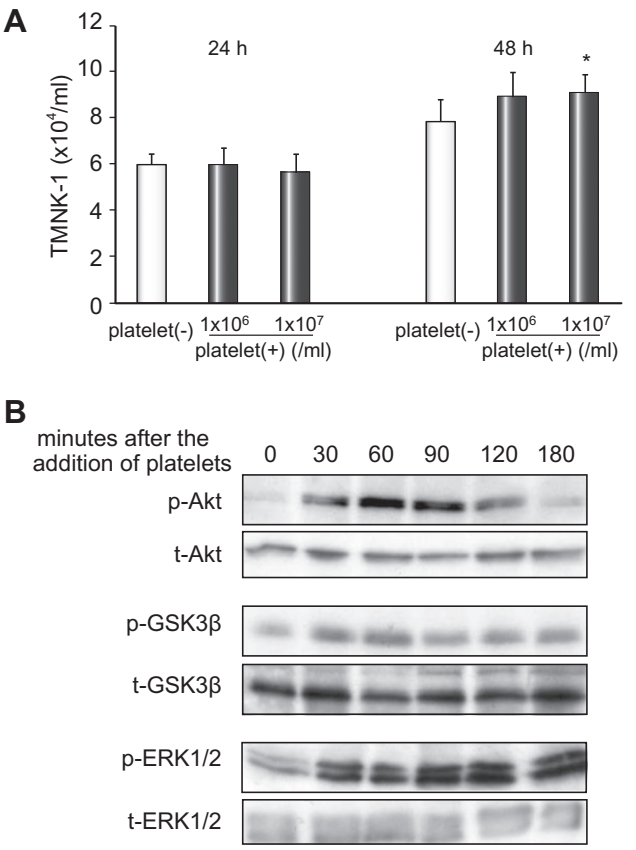


Fig. 1. (A) Effects of platelets on TMNK-1 proliferation. The effect of platelets on TMNK-1 proliferation was evaluated by counting TMNK-1 cells cocultured with platelets for 24 and 48 h. Data are expressed as means ± SD. $n = 4$ in each group. *p <0.05 versus the platelet (−) group. (B) Signal transduction of TMNK-1. To investigate the mechanism of platelet-induced TMNK-1 proliferation, TMNK-1 cells were harvested at 0, 30, 60, 90, 120, and 180 min after the addition of platelets, and activation of the Akt, ERK1/2, and STAT3 pathways was examined by Western blotting.

Effects of S1P on excretion of IL-6 from TMNK-1

Th concentration of IL-6 was significantly increased in the group administered with S1P compared to those without S1P (Fig. 4).

Necessity of contact with platelets for excretion of IL-6 from TMNK-1

In the group in which the platelets and TMNK-1 were cultured separately, no significant increase of IL-6 was observed (Fig. 5).

Effects of the supernatants of TMNK-1 on hepatocyte proliferation

BrdU uptake of the hepatocytes in the group administered with the supernatant co-cultured with platelets was significantly higher than in the group administered with the supernatant cultured without platelets (Fig. 6A).

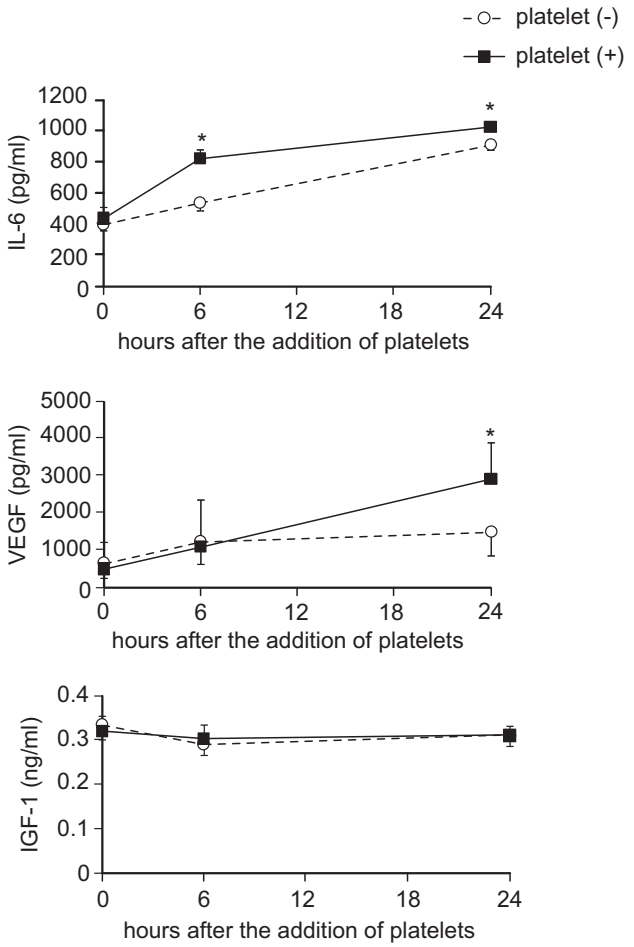


Fig. 2. Assay of HGF, IL-6, VEGF, and IGF-1 in the supernatant of cultured TMNK-1. The amounts of HGF, IL-6, VEGF, and IGF-1 in the supernatant of TMNK-1 were measured at 0, 6, and 24 h after the addition of platelets. Data are expressed as means ± SD. $n = 6$ in each group. $^*p < 0.05$ versus the platelet (−) group. IL-6, interleukin-6; VEGF, vascular endothelial growth factor; IGF-1, insulin-like growth factor 1.

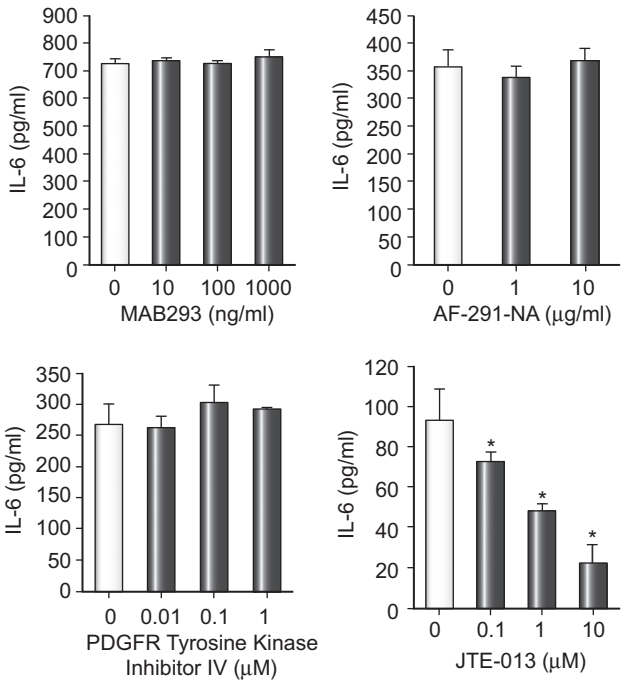


Fig. 3. Effects of inhibitors on excretion of IL-6 from TMNK-1 with platelets. To determine which platelet component had an effect on excretion of IL-6 from TMNK-1, we inhibited the excreted soluble factors using neutralizing antibodies or receptor antibodies, and evaluated the excretion of IL-6 from TMNK-1. Anti-human VEGF Antibody MAB293, Anti-human IGF-I Antibody AF-291-NA, PDGFR Tyrosine Kinase Inhibitor IV, and a specific antagonist for S1P2 receptors JTE-013 were added to TMNK-1 or platelets. TMNK-1 cells were then cocultured with platelets for 6 h, and the amounts of IL-6 in the supernatant of TMNK-1 were measured. Data are expressed as means ± SD. $n = 4$ in each group. $^*p < 0.05$ versus the inhibitor (−) group. IL-6, interleukin-6.

Signal transduction of primary hepatocytes cultured with supernatant of TMNK-1 activated by platelets

The Akt pathway was activated within 5 min, the ERK1/2 pathway within 10 min, and the STAT3 pathway within 5 min after the addition of the supernatant of TMNK-1 activated by platelets (Fig. 6B).

Effects of IL-6 in the supernatants of TMNK-1 on hepatocyte proliferation

BrdU uptake of the hepatocytes was significantly decreased in the group administered with anti-IL-6 antibody MAB206 compared to those without MAB206 (Fig. 6C).

Discussion

Platelets have an important role in a various stress mechanisms of the liver and we previously reported that platelets promote hepatocyte proliferation [12,13,16–23]. Recently, Lesurtel et al. reported that platelet-derived serotonin mediates liver regeneration *in vivo* [27]. However, there are no prior studies regarding the relationship between human platelets, LSEC, and hepatocytes in liver regeneration. In this study, we focused on the role of LSECs and hepatocytes in liver regeneration in addition to platelets. We used human platelets and human primary hepatocytes; we did not use primary LSECs but the immortalized cell line TMNK-1 as LSECs. Using cocultured LSECs with human platelets revealed that platelets cause proliferation of LSECs and induce the excretion of IL-6, VEGF, and IGF-1 from LSECs through activating Akt and ERK1/2 signals. The excretion of IL-6 from LSECs requires direct contact between platelets and LSECs. The S1P of platelets are responsible for producing IL-6 in LSEC. IL-6 from LSECs causes proliferation of hepatocytes by activating STAT3. The results in this study indicate that platelets induced hepatocyte proliferation through LSEC activation.

The PI3/Akt pathway is activated by growth factors and is known as a survival signaling pathway [28]. GSK3β is located downstream of Akt and plays a critical role in liver regeneration by regulating cell size [29]. It was reported that Akt of rat LSEC was phosphorylated after the addition of VEGF [30] or PDGF [31]. The ERK1/2 pathway is also activated by growth factors and involved in growth and differentiation [32]. There are no reports indicating that any growth factors activate the ERK1/2 pathway of LSEC. It was reported that the ERK1/2 of human umbilical vein endothelial cells was phosphorylated after the addition of VEGF [33]. In this study, the Akt and ERK1/2 pathways

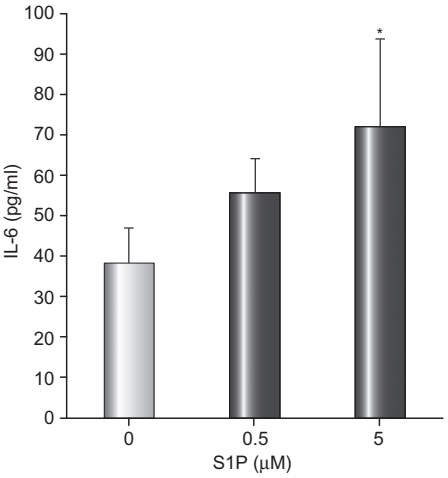


Fig. 4. Effects of S1P on excretion of IL-6 from TMNK-1. To determine whether S1P had an effect on excretion of IL-6 from TMNK-1, TMNK-1 cells were cultured with S1P for 6 h, and the amounts of IL-6 in the supernatant of TMNK-1 were measured. Data are expressed as means ± SD. $n = 4$ in each group. $^*p < 0.05$ versus the S1P (−) group. IL-6, interleukin-6; S1P, sphingosine 1-phosphate.

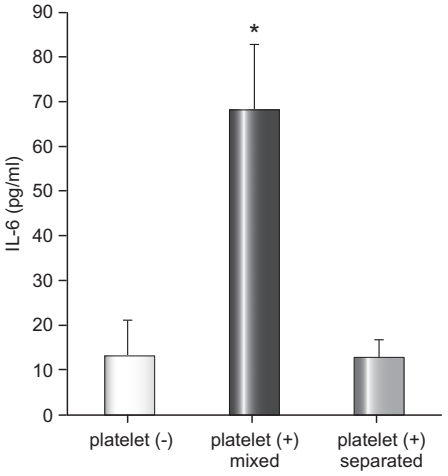


Fig. 5. Necessity of contact with platelets for excretion of IL-6 from TMNK-1. To investigate the necessity of direct contact between platelets and TMNK-1, TMNK-1 and platelets were cocultured using a cell culture insert for 6 h, and excretion of IL-6 from TMNK-1 was measured. Data are expressed as means ± SD. $n = 4$ in each group. $^*p < 0.05$ versus the platelet (+) separated group. IL-6, interleukin-6.

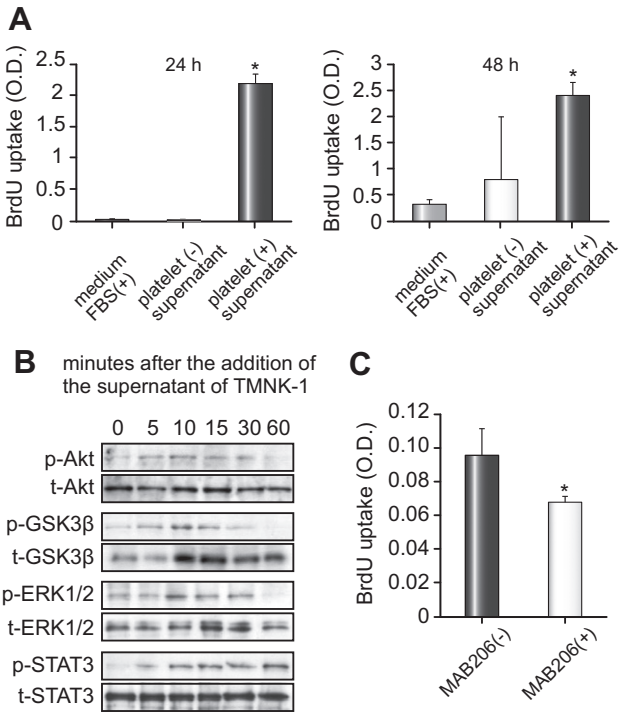


Fig. 6. Effects of the supernatants of TMNK-1. (A) Effects of the supernatants of TMNK-1 on hepatocyte proliferation. Primary hepatocytes were cultured with the supernatants of TMNK-1, and after 24 and 48 h incubation, BrdU uptake was measured. Data are expressed as means ± SD. $n = 8$ in each group. $^*p < 0.05$ versus the platelet (−) supernatant group. (B) Signal transduction of primary hepatocytes cultured with the supernatant of TMNK-1 activated by platelets. To investigate the mechanism of effects of the supernatants of TMNK-1 on hepatocyte proliferation, hepatocytes were harvested at 0, 5, 10, 15, 30, and 60 min after the addition of the supernatants of TMNK-1 with platelets, and activation of the Akt, ERK1/2, and STAT3 pathways was examined by Western blotting. (C) Effects of IL-6 in the supernatants of TMNK-1 on hepatocyte proliferation. Primary hepatocytes were cultured with the supernatant of TMNK-1, which was pre-incubated with or without anti-IL-6 antibody MAB206, and after 24 h incubation, BrdU uptake was measured. Data are expressed as means ± SD. $n = 4$ in each group. $^*p < 0.05$ versus the MAB206 (−) supernatant group. IL-6, interleukin-6.

of TMNK-1 were activated after the addition of platelets and the proliferation of TMNK-1 was induced. This effect may be caused by some growth factors or S1P. Platelets contain VEGF, PDGF, and S1P; therefore, they are likely involved in the activation of the Akt and ERK1/2 pathways of TMNK-1.

IL-6 is known to play a crucial role in parenchymal hepatocyte proliferation by activating STAT3 [34]. In this study, concentrations of IL-6 in the supernatant of TMNK-1 were significantly increased in the groups with platelets compared to the groups without platelets. Activation of the STAT3 pathway of hepatocytes as well as Akt and ERK1/2 activation and promotion of DNA synthesis were presumably due to IL-6 in the supernatants of TMNK-1. VEGF does not have a strong effect on hepatocyte proliferation [35]. Therefore, it is likely that IL-6, excreted from SEC, mainly contributed to hepatocyte proliferation rather than VEGF.

S1P belongs to lipid mediators and has been revealed to regulate diverse biological processes [24]. S1P is excreted in large amounts from activated platelets [36]. It was reported that S1P promotes DNA synthesis of LSECs and protects LSECs from ethanol-induced apoptosis through endothelial NO synthase activation in rat [30]. It was also reported that S1P causes the excretion of IL-6 from other cells, such as human airway smooth muscle cells [37] and immature human dendritic cells [38]. When platelets and TMNK-1 were separated, no significant increment of IL-6 was observed. The concentration of IL-6 was significantly decreased in the supernatant of TMNK-1 cocultured with platelets after the addition of S1P inhibitor. On the other hand, the concentration of IL-6 was significantly increased after the addition of S1P alone. These results indicate that direct contact between platelets and LSECs triggered the excretion of S1P from the platelets, S1P derived from platelets induced the excretion of IL-6 from LSECs (Fig. 7). Also, the DNA synthesis of hepatocytes was then significantly promoted by stimulating IL-6 excretion

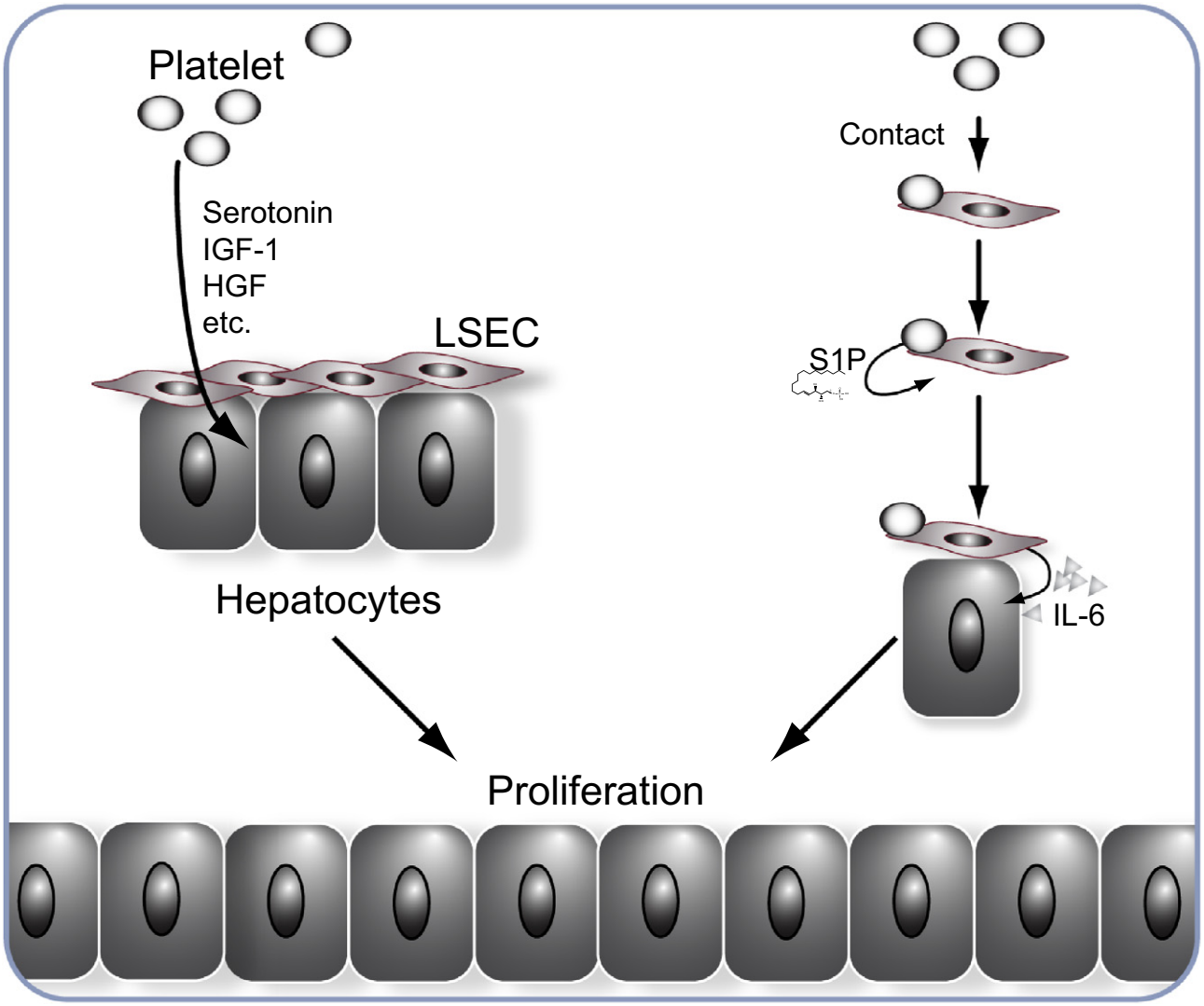


Fig. 7. Scheme for liver regeneration promoted by LSECs and platelets. It was reported that platelets promote hepatocyte proliferation. Our results indicate that direct contact between platelets and LSECs triggered the excretion of S1P from the platelets, which then caused the excretion of IL-6 from LSECs. IL-6 from LSECs promoted hepatocyte proliferation. IGF-1, insulin-like growth factor 1; HGF, hepatocyte growth factor; LSEC, liver sinusoidal endothelial cell; S1P, sphingosine 1-phosphate; IL-6, interleukin-6.

from LSECs. Platelets contribute to hepatocyte proliferation with activation of LSECs through S1P and IL-6.

Conflict of Interest

The authors who have taken part in this study do not have a relationship with the manufacturers of the drugs involved either in the past or present and did not receive funding from the manufacturers to carry out their research. The authors received support from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT).

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References

[1] Shirabe K, Shimada M, Gion T, Hasegawa H, Takenaka K, Utsunomiya T. Postoperative liver failure after major hepatic resection for hepatocellular carcinoma in the modern era with special reference to remnant liver volume. J Am Coll Surg 1999;188:304–309.
[2] Topal B, Kaufman L, Aerts R, Penninckx F. Patterns of failure following curative resection of colorectal liver metastases. Eur J Surg Oncol 2003;29:248–253.
[3] Khan AZ, Mudan SS. Liver regeneration: mechanisms, mysteries and more. ANZ J Surg 2007;77:9–14.
[4] Michalopoulos GK, DeFrances MC. Liver regeneration. Science 1997;276:60–66.
[5] Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. Cell 2007;129:1261–1274.
[6] Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 2002;298:1911–1912.
[7] Malik R, Selden C, Hodgson H. The role of non-parenchymal cells in liver growth. Semin Cell Dev Biol 2002;13:425–431.
[8] Ping C, Xiaoling D, Jin Z, Jiahong D, Jiming D, Lin Z. Hepatic sinusoidal endothelial cells promote hepatocyte proliferation early after partial hepatectomy in rats. Arch Med Res 2006;37:576–583.

[9] Rozman P, Bolta Z. Use of platelet growth factors in treating wounds and soft-tissue injuries. Acta Dermatovenerol Alpina Panonica Adriatica 2007;16:156–165.
[10] Gruber R, Varga F, Fischer MB, Watzek G. Platelets stimulate proliferation of bone cells: involvement of platelet-derived growth factor, microparticles and membranes. Clin Oral Implants Res 2002;13:529–535.
[11] Carter CA, Jolly DG, Worden CE, Hendren DG, Kane CJM. Platelet-rich plasma gel promotes differentiation and regeneration during equine wound healing. Exp Mol Pathol 2003;74:244–255.
[12] Clavien P. Liver regeneration: a spotlight on the novel role of platelets and serotonin. Swiss Med Wkly 2008;138:361–370.
[13] Nakano Y, Kondo T, Matsuo R, Hashimoto I, Kawasaki T, Kohno K, et al. Platelet dynamics in the early phase of postischemic liver in vivo. J Surg Res 2008;149:192–198.
[14] Johnson RJ, Alpers CE, Pritzl P, Schulze M, Baker P, Pruchno C. Platelets mediate neutrophil-dependent immune complex nephritis in the rat. J Clin Invest 1988;82:1225–1235.
[15] Sindram D, Porte RJ, Hoffman MR, Bentley RC, Clavien PA. Platelets induce sinusoidal endothelial cell apoptosis upon reperfusion of the cold ischemic rat liver. Gastroenterology 2000;118:183–191.
[16] Murata S, Ohkohchi N, Matsuo R, Ikeda O, Myronovych A, Hoshi R. Platelets promote liver regeneration in early period after hepatectomy in mice. World J Surg 2007;31:808–816.
[17] Matsuo R, Ohkohchi N, Murata S, Ikeda O, Nakano Y, Watanabe M, et al. Platelets strongly induce hepatocyte proliferation with IGF-1 and HGF in vitro. J Surg Res 2008;145:279–286.
[18] Hoshi R, Murata S, Matsuo R, Myronovych A, Hashimoto I, Ikeda H. Freeze–dried platelets promote hepatocyte proliferation in mice. Cryobiology 2007;55:255–260.
[19] Murata S, Matsuo R, Ikeda O, Myronovych A, Watanabe M, Hisakura K, et al. Platelets promote liver regeneration under conditions of kupffer cell depletion after hepatectomy in mice. World J Surg 2008;32: 1088–1096.
[20] Myronovych A, Murata S, Chiba M, Matsuo R, Ikeda O, Watanabe M, et al. Role of platelets on liver regeneration after 90% hepatectomy in mice. J Hepatol 2008;49:363–372.
[21] Ikeda O, Ozaki M, Murata S, Matsuo R, Nakano Y, Watanabe M, et al. Autonomic regulation of liver regeneration after partial hepatectomy in mice. J Surg Res 2009;152:218–223.
[22] Watanabe M, Murata S, Hashimoto I, Nakano Y, Ikeda O, Aoyagi Y, et al. Platelets contribute to the reduction of liver fibrosis in mice. J Gastroenterol Hepatol 2009;24:78–89.
[23] Murata S, Hashimoto I, Nakano Y, Myronovych A, Watanabe M, Ohkohchi N. Single administration of thrombopoietin prevents progression of liver fibrosis and promotes liver regeneration after partial hepatectomy in cirrhotic rats. Ann Surg 2008;248:821–828.
[24] Spiegel S, Milstien S. Sphingosine-1-phosphate: an enigmatic signalling lipid. Nat Rev Mol Cell Biol 2003;4:397–407.
[25] Matsumura T, Takesue M, Westerman KA, Okitsu T, Sakaguchi M, Fukazawa T, et al. Establishment of an immortalized human-liver endothelial cell line with SV40T and hTERT. Transplantation 2004;77:1357–1365.
[26] Seglen PO. Preparation of isolated rat liver cells. Methods Cell Biol 1976;13:29–83.
[27] Lesurtel M, Graf R, Aleil B, Walther DJ, Tian Y, Jochum W, et al. Platelet-derived serotonin mediates liver regeneration. Science 2006;312:104–107.
[28] Ozaki M, Haga S, Zhang HQ, Irani K, Suzuki S. Inhibition of hypoxia/reoxygenation-induced oxidative stress in HGF-stimulated antiapoptotic signaling: role of PI3-K and akt kinase upon rac1. Cell Death Differ 2003;10:508–515.
[29] Haga S, Ogawa W, Inoue H, Terui K, Ogino T, Igarashi R, et al. Compensatory recovery of liver mass by akt-mediated hepatocellular hypertrophy in liver-specific STAT3-deficient mice. J Hepatol 2005;43:799–807.
[30] Zheng D, Kitamura T, Ikejima K, Enomoto N, Yamashina S, Suzuki S, et al. Sphingosine 1-phosphate protects rat liver sinusoidal endothelial cells from ethanol-induced apoptosis: role of intracellular calcium and nitric oxide. Hepatology 2006;44:1278–1287.
[31] Liu S, Premont RT, Kontos CD, Huang J, Rockey DC. Endothelin-1 activates endothelial cell nitric-oxide synthase via heterotrimeric G-protein bet-agamma subunit signaling to protein jinase B/Akt. J Biol Chem 2003;278:49929–49935.
[32] Pearson G, Robinson F, Gibson TB, Xu BE, Karandikar M, Berman K. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev 2001;22:153–183.
[33] Xu Z, Maiti D, Kisiel W, Duh EJ. Tissue factor pathway inhibitor-2 is upregulated by vascular endothelial growth factor and suppresses growth factor-induced proliferation of endothelial cells. Arterioscler Thromb Vasc Biol 2006;26:2819–2825.
[34] Levy DE, Lee C. What does Stat3 do? J Clin Invest 2002;109:1143–1148.
[35] LeCouter J, Moritz DR, Li B, Phillips GL, Liang XH, Gerber H, et al. Angiogenesis-independent endothelial protection of liver: role of VEGFR-1. Science 2003;299:890–893.
[36] Yatomi Y, Ohmori T, Rile G, Kazama F, Okamoto H, Sano T, et al. Sphingosine 1-phosphate as a major bioactive lysophospholipid that is released from platelets and interacts with endothelial cells. Blood 2000;96:3431–3438.
[37] Sindram D, Porte RJ, Hoffman MR, Bentley RC, Clavien PA. Synergism between platelets and leukocytes in inducing endothelial cell apoptosis in the cold ischemic rat liver: a kupffer cell-mediated injury. FASEB J 2001;15:1230–1232.
[38] Oz-Arslan D, Rüscher W, Myrtek D, Ziemer M, Jin Y, Damaj BB, et al. IL-6 and IL-8 release is mediated via multiple signaling pathways after stimulating dendritic cells with lysophospholipids. J Leukoc Biol 2006;80:287–297.