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## Title

Platelets strongly induce hepatocyte proliferation with IGF-1 and HGF *in vitro*<sup>1</sup>

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## Running title

The role of platelet in hepatocyte proliferation

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## Subject category

Gastrointestinal

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**Background.** It is well known that platelets have a thrombotic effect. However, not only in hemostasis, platelets also play an important role in wound healing and tissue regeneration. Platelets have been reported to accumulate in the liver and promote liver regeneration after an extended hepatectomy, but the mechanism is unclear. The present study was designed to clarify the mechanism by which platelets have a direct proliferative effect on hepatocytes *in vitro*.

**Materials and Methods.** Hepatocytes obtained from male BALB/c mice by collagenase digestion and immortalized hepatocytes (TLR2) were used. To elucidate the mechanism of platelets' proliferative effect, DNA synthesis of hepatocytes were measured under various conditions and the related cellular signals were analyzed. Chromatographic analysis was also performed to clarify which elements of platelet have mitogenic activity.

**Results.** DNA synthesis significantly increased in the hepatocytes cultured with platelets ( $p < 0.001$ ). However, when the platelets and hepatocytes were separated, the platelets did not have a proliferative effect. Whole disrupted platelets, the supernatant fraction, and fresh isolated platelets had a similar proliferative effect, while the membrane fraction did not. After the addition of platelets, both Akt and ERK1/2 were activated, but STAT3 was not activated. Some mitogenic fractions were obtained from the platelet extracts by gel exclusion chromatography; the fractions were rich in HGF and IGF-1.

**Conclusions.** Direct contact between platelets and hepatocytes was necessary for the proliferative effect. The direct contact initiated signal transduction involved in growth factor activation. HGF, VEGF, and IGF-1 rather than PDGF mainly contributed to hepatocyte proliferation.

**Key Words:** platelets, hepatocytes, growth factors, cytokines, direct contact, signal transduction, STAT3, Akt, ERK1/2, chromatographic analysis

**Abbreviations:** ERK, extracellular signal-regulated kinases, STAT, signal transducer and activator of transcription

## INTRODUCTION

The precise mechanism of liver regeneration is not well understood. In the last two decades, many studies have focused on hepatotropic factors that could prevent hepatic failure after an extended hepatectomy or other types of liver injury [1, 2]. However, no such substances have been developed for clinical use. It is well known that platelets have a thrombotic effect. Platelets contain not only proteins needed for hemostasis, but also many growth factors, such as PDGF, HGF, VEGF, EGF, and TGF- $\beta$  [3-8], that are required for tissue regeneration [9-11]. During wound healing, platelets have been reported to come into contact with collagen and other extracellular matrix elements at the site of injury [9]. This direct contact triggers the release of clotting factors, essential growth factors, and cytokines [9]. During liver injury, platelets have been shown to actively translocate into Disse's spaces in response to lipopolysaccharides [12-14]. However, there are a few studies concerning the relationship between platelets and liver regeneration [15, 25]. We recently reported that platelets accumulated in the liver of mice immediately after a 70% hepatectomy and in the electron microscopic finding, the migration of platelets from sinusoidal spaces into Disse's spaces through the fenestration of the sinusoidal endothelial cells was recognized. In the early period after hepatectomy regeneration of residual liver occurred earlier in platelet-increased mice than in platelet-depleted mice [15]. Based on these findings of our previous study, we hypothesized that platelets could sense neighboring hepatocytes and that direct contact of platelets and hepatocytes was necessary for the release of various signals, such as inflammatory cytokines and growth factors, thereby promoting liver regeneration.

In the present study, hepatocytes were co-cultured with/without platelets and DNA synthesis was measured to clarify the role of platelets in hepatocyte proliferation *in vitro*. We investigated whether direct contact between platelets and hepatocytes was necessary for the proliferative effect and which platelet components i.e., membrane, soluble factors, or specific growth factors are required for hepatocyte proliferation. We also analyzed the cellular signals, such as STAT3, Akt, and ERK1/2 pathways, which are involved in platelet-induced hepatocyte proliferation.

## MATERIALS AND METHODS

### *Animals*

Seven-week-old male BALB/c mice (Clea, Tokyo, Japan), weighing 20 to 25 g each were used for hepatocyte isolation and platelet collection. The animals were housed, 5 per cage, in an animal room under a 12 h light/12 h dark cycle and had free access to water and chow. The animals were handled according to our institution's guidelines.

### *Isolation and culture of mice hepatocytes*

Hepatocytes were prepared using the Seglen collagenase perfusion technique [16, 17].

10 The isolated parenchymal cell suspension had a viability between 90 and 95% on the Trypan blue exclusion test. The hepatocytes were cultured in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C in Williams' E medium (Sigma, St. Louis, MO) supplemented with 200 µM l-glutamine (Invitrogen, Grand Island, NY), 10% FBS (Hyclone, Road Logan, UT), 100 µM dexamethasone (Sigma), 1 µg/ml insulin (Sigma), 1% penicillin and streptomycin (Invitrogen).

### *Hepatocyte cell line TLR2*

TLR2 is a hepatocyte cell line derived from C57Bl6 mice established by the transfection of SV-40 that proliferates *in vitro* [18]. TLR2 were cultured in a humidified atmosphere containing 5% CO<sub>2</sub> at 33°C in RITC80-7 medium (Iwaki, Tokyo, Japan) supplemented with 1  
20 µg/ml insulin (Sigma), 10 µg/ml transferrin (Sigma), 10 µg/ml EGF (Roche Diagnostics, Hague Road, IN) and 2% FBS (Hyclone).

### *Preparation of washed platelets*

Blood was obtained from the mice by cardiac puncture under ether anesthesia. Platelet-rich plasma (PRP) was obtained by centrifuging anticoagulated blood containing acid citrate dextrose (ACD) in a 1:4 volume ratio at 200×g and 24°C for 10 min. The platelets were washed twice by centrifugation of PRP at 1000×g and 4°C for 15 min and resuspended in citrate buffer (120 mM NaCl, 4.26 mM NaHPO<sub>4</sub>, 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 serum-free medium.

### *Disruption of platelets and isolation of fractions*

The platelets were disrupted by 5 freeze-thawing cycles. The homogenate was centrifuged at 15000xg for 60 min; the resultant supernatant was used as the platelet soluble fraction, and the pellet was used as the membrane fraction.

### *Incorporation of [<sup>3</sup>H]-methyl-thymidine*

Primary cultured hepatocytes were radiolabeled with [<sup>3</sup>H]-methyl-thymidine (Amersham Bioscience, Buckinghamshire, UK). Briefly, hepatocytes ( $2.0 \times 10^5$  cells/well) were seeded in 10 6-well culture plates with/without platelets or fractions and grown for 24 hours; 3  $\mu$ Ci of [<sup>3</sup>H]-methyl-thymidine were added to each well. After 48 hours of incubation, the cells were harvested; [<sup>3</sup>H]-methyl-thymidine incorporation was measured using a liquid scintillation counter (LS-6500, Beckman Coulter, Fullerton, CA).

### *BrdU assay*

The dose-dependent effect of platelets on hepatocyte proliferation was evaluated using the BrdU assay kit (Roche Diagnostics GmbH, Penzberg, Germany). Briefly, primary hepatocytes ( $5 \times 10^3$  in serum-free Williams' E medium) were seeded in 96-well plates. Then, platelets suspended in serum-free Williams' E medium (50  $\mu$ l/well) at different densities 20 ( $1 \times 10^5$ /ml,  $1 \times 10^6$ /ml,  $1 \times 10^7$ /ml,  $2 \times 10^7$ /ml, and  $1 \times 10^8$ /ml) were added to each well. After incubation at 37°C for 48 hours, BrdU uptake was measured.

### *Co-culture system*

For separated co-culture, hepatocytes and platelets were co-cultured using a cell culture insert (pore size; 0.3  $\mu$ m, Becton Dickinson, Bedford, MA), and hepatocyte [<sup>3</sup>H]-methyl-thymidine uptake was measured as described above.

To determine the importance of direct contact, another co-culture system was designed. TLR2 cells were plated at a density of  $1.0 \times 10^5$  cells/well in 6-well culture plates and incubated for 4 hours; thereafter, cell culture inserts (pore size; 0.3  $\mu$ m) were placed in each well. Four groups

were prepared as follows: platelet (-) group, neither cells nor platelets were seeded in the upper chamber (Fig.4A); mixed co-culture group (co-mix),  $5 \times 10^7$  platelets were seeded in the lower chamber with the hepatocytes, but neither hepatocytes nor platelets were seeded in the upper chamber (Fig.4B); separated co-culture group (co-sep), platelets were seeded in the upper chamber, and hepatocytes were seeded in the lower chamber (Fig.4C); upper mix group, hepatocytes were seeded in the upper chamber and overlaid with platelets, while hepatocytes in the lower chamber were cultured without contacting the hepatocytes and platelets in the upper chamber (Fig.4D); thrombin-stimulated group, platelets in the upper chamber were stimulated with murine thrombin (1 u/ml) to release soluble factors (Fig.4E). After incubation for 72 hours, the TLR2 cells in the lower chamber were counted.

#### *Western blot analysis*

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, and phospho-ERK1/2 (Cell signaling, Beverly, MA).

#### *Gel exclusion chromatography of platelet supernatants*

Chromatographic analysis was performed. Platelet supernatant (500  $\mu$ l) corresponding to  $1 \times 10^{10}$  platelets/ml was applied to a Superdex G-200 (Amersham Bioscience) column equilibrated with PBS and eluted at a flow rate of 30 ml/h at 4°C. One-ml fractions were collected and assayed for their mitogenic activity by BrdU uptake and for their concentration of growth factors (PDGF, HGF, VEGF, IGF-1, and EGF) by Western blotting.

#### *Statistical analysis*

Results are expressed as the mean  $\pm$  S.D. Statistical analyses were performed with one way ANOVA; p-values less than 0.05 were considered statistically significant.

## RESULTS

### *Proliferative effect of platelets on hepatocytes*

In order to evaluate the platelets' proliferative effect, hepatocytes were co-cultured with platelets. Then, after 72-h incubation, [<sup>3</sup>H]-methyl-thymidine uptake and BrdU uptake were measured. Thymidine uptake in the platelet (+) group was significantly higher than in the platelet (-) group ( $p < 0.001$ ) (Fig.1A). The degree of BrdU uptake depended on the platelet concentration ( $p < 0.001$ ) (Fig.1B).

### *Necessity of contact with platelets for hepatocyte proliferation to occur*

10 To investigate the necessity of direct contact between platelets and hepatocytes, we prepared a separated co-culture group in which the hepatocytes were cultured in a chamber that separated them from platelets by a permeable membrane with 0.3- $\mu$ m pores. DNA synthesis in the hepatocytes was measured using [<sup>3</sup>H]-methyl-thymidine uptake. In the group in which the platelets and hepatocytes were separated, no significant proliferative effect was observed (Fig.2).

### *Effective component of platelets*

To determine which platelet component had an effect on hepatocyte proliferation, the mitogenic activity of whole disrupted platelets, the soluble fraction, and the membrane fraction was evaluated as described above. Whole disrupted platelets and the soluble fraction had a  
20 significant proliferative effect ( $p < 0.0001$ ); the membrane fraction did not (Fig.3). The effect of the membrane fraction on hepatocyte proliferation was confirmed using another method. Membrane lipids were extracted using the methanol/chloroform extraction method, added to cultured hepatocytes, and then DNA synthesis was measured. The membrane lipid fraction had no proliferative effect (data not shown).

### *The characteristics of direct contact between hepatocytes and platelets*

To further elucidate the characteristics of the direct contact, 4 groups of separated co-culture were prepared as follows: the platelet (-) group, the mixed co-culture group (co-mix), the separated co-culture group (co-sep), and the upper mix group (upper mix)(Fig.4A-D). In a

same manner, a thrombin-stimulated group was prepared (Fig.4E). TLR2 cells in the lower chamber were counted after 72-h incubation. In the upper mix group, platelets induced the proliferation of hepatocytes in the lower chamber ( $p<0.001$ ). This result indicates that, upon direct contact with hepatocytes, platelets released soluble factors that induced hepatocyte proliferation. This effect in the upper mix group was of the same magnitude as in the co-mix group. A proliferative effect was also observed in the thrombin-stimulated group, although no direct contact between platelets and hepatocytes occurred (Fig.4F).

### *Signal transduction*

10 To investigate the mechanism of platelet-induced hepatocyte proliferation, we analyzed the hepatocyte signals that were activated by platelets. The phosphorylation of Akt, ERK1/2, and STAT3 was analyzed in hepatocytes stimulated by platelets for up to 60 min (Fig.5). The Akt pathway was activated within 1 min and ERK1/2 pathway was activated within 10 min after the addition of platelets; the STAT3 pathway was not. The Akt and ERK1/2 pathways are well known as downstream signals for growth factor receptors.

### *Gel exclusion chromatography of platelet extract*

20 To determine which element of the platelet soluble factor exerted the proliferative effect, platelet extracts were gel-excluded into 18 fractions. Mitogenic activity of each fraction was evaluated on BrdU assay as described above. Mitogenic activity was strong in fractions 1, 2, 5-7, and 14-17 (Fig.6A). To identify the fraction, Western blotting was performed using the following antibodies: anti-HGF, PDGF, VEGF, IGF-1, EGF antibody (R&D, Minneapolis, MN). Fraction 4-6 were rich in HGF, fractions 5-7 were rich in VEGF, fractions 7-9 were rich in PDGF, fraction 14-17 were rich in IGF-1, while no fractions contained EGF. In addition, hepatocyte signals were analyzed in response to growth factors, such as HGF, PDGF, IGF-1, VEGF, and serotonin, that are present in platelets. HGF, IGF-1, and VEGF strongly activated the Akt and the ERK1/2 pathways, however PDGF and serotonin with/without EGF-pretreatment did not (Fig.6B).

## DISCUSSION

Platelets are characteristically activated at the site of injury to form a physical barrier to limit blood loss and accelerate the formation of thrombin to intensify the coagulation process [19]. Platelets also release local mediators and interact with leukocytes and endothelial cells to modulate the inflammatory reaction [19]. Platelets are involved not only in hemostasis and inflammation, but also in wound healing and tissue repair [9]. The liver is a highly vascularized organ, and accumulation of platelets occurs immediately after hepatectomy or other types of liver injury, such as ischemic reperfusion or LPS administration [12-15]. In mice under condition of platelets increment, liver regeneration was accelerated after hepatectomy [15].

10 From these reports, platelets appear to be strongly associated with tissue repair in injured livers and in liver regeneration. In this study, we investigated the direct proliferative effect of platelets on hepatocytes *in vitro*, i.e., by adding platelets to cultured hepatocytes and measuring hepatocyte proliferation. DNA synthesis of hepatocyte significantly increased in the platelet (+) group compared to the platelet (-) group and direct contact of platelets and hepatocytes was essential for hepatocyte proliferation, therefore, platelets had a significant effect on hepatocyte proliferation.

It has also been reported that platelets actively translocate into Disse's spaces. Flattened sinusoidal endothelial cells are fenestrated; the diameter of the fenestrae is smaller than that of platelets. In response to hepatectomy, these fenestrae immediately become larger [20, 21], and  
20 platelets actively translocate into Disse's spaces through the fenestrae, thus coming into direct contact with hepatocytes [15]. The active translocation of platelets into Disse's spaces also occurs in response to lipopolysaccharides [12, 14]. To determine role of platelets' direct contact with hepatocytes, we investigated whether platelets had a direct effect on hepatocyte proliferation using co-culturing chamber where platelets and hepatocytes were separated by a permeable membrane. When the platelets and hepatocytes were separated, the platelets had no proliferative effect. These results clearly demonstrated that direct contact between platelets and hepatocytes was essential for inducing hepatocyte proliferation.

Platelets contain  $\alpha$ -granules and dense granules, and  $\alpha$ -granules contain several growth factors, such as PDGF, VEGF, IGF, and some cytokines [9, 22, 23]. Dense granules contain ADP,

ATP, and serotonin [9, 22, 23]. To clarify which platelet component i.e., membrane or soluble fraction, was responsible for the proliferative effect, we disrupted the platelet's membrane and obtained the membrane fraction and the soluble fraction. Each fraction was added to cultured hepatocytes, and DNA synthesis was measured. The soluble fraction had a proliferative effect on hepatocytes. On the other hand, the membrane fraction did not have that effect. These results of our study indicate that direct contact was essential, though the membrane itself did not play a role in hepatocyte proliferation. By contact with collagen and other extracellular matrixes, platelets are known to become activated and release essential growth factors and cytokines for wound healing [9]. From previous reports and our results, it could be strongly suspected that direct contact between platelets and hepatocytes is essential to trigger the release of the soluble factors that are necessary for liver regeneration.

Hepatocytes have a mitogenic response to various growth factors and cytokines, such as HGF, IL-6, TNF- $\alpha$ , EGF, TGF- $\alpha$  and PDGF [24]. The  $\alpha$ -granules of platelets contain effective growth factors, such as PDGF, HGF, IGF, and VEGF. In this study, to elucidate the characteristics of cell to cell contact and the release of soluble factors from platelets, a particular separated co-culture model was used. In the upper mix co-culture group the platelets had a strong proliferative effect on the hepatocytes in the lower chamber, even though they were separated from the platelets and hepatocytes in the upper chamber. This result clearly indicates that direct contact between platelets and hepatocytes triggered the release of soluble factors from the platelets, thereafter which then caused a proliferative effect on the hepatocytes in the lower chamber. In addition, we stimulated platelets with thrombin, which is known to stimulate the release of  $\alpha$ -granules. In such a condition, hepatocytes in the lower chamber were recognized to proliferate, even though they were separated from the thrombin-stimulated platelets. To further confirmation of the effect of platelets' soluble factors, we inhibited the released some soluble factors, i.e., IGF-1 and HGF using neutralizing antibodies and evaluated hepatocyte proliferation. Anti-IGF-1 and anti-HGF antibodies (R&D) significantly inhibited hepatocyte proliferation stimulated by platelet soluble factors (data not shown). These results in our study indicate that platelets induced hepatocyte proliferation by stimulation of platelet as well as via soluble factors with direct contact.

It has been reported that some pathways of signal transduction are highly associated with liver regeneration. The STAT3 pathway which is activated by cytokines such as IL-6 is known to play a crucial role in cell proliferation [25, 26]. The Akt pathway which is activated by growth factors is known as a survival signaling pathway [27, 28]. The ERK1/2 pathway which is activated by growth factors is involved in growth and differentiation [29]. We previously reported that the Akt and ERK1/2 pathways were activated immediately after hepatectomy in mice that had a higher number of platelets compared to platelet-depleted mice [15]. In the present study, the Akt and ERK1/2 pathways were also activated immediately after the addition of platelets, but the STAT3 pathway was not activated. These results indicate that platelets induce hepatocyte proliferation via growth factors.

In a recent study, it was reported that platelet-derived serotonin was involved in the initiation of liver regeneration [30]. However, the mechanism is not clarified yet. In our study we obtained mitogenic fractions that contained HGF, VEGF, and IGF-1 by gel exclusion chromatography. As a result, IGF-1 rich fraction was revealed to be the most effective growth factor contained in platelets. The hepatocyte proliferative signals stimulated by soluble platelet factors were also analyzed. HGF, VEGF, and IGF-1 activated the Akt and ERK1/2 pathways to the same degree as fresh isolated platelets, but PDGF, serotonin, and serotonin with EGF pretreatment did not. Furthermore, we examined the role of the Akt pathway in hepatocyte proliferation. In the presence of LY294002 which is PI3k inhibitor and inhibits the activation of the Akt pathway, platelets did not exert a proliferative effect on hepatocytes (data not shown). These results indicate that the Akt pathway is one of the important signals and that the activators of the Akt pathway are key molecules involved in the direct proliferative effect of platelets.

In conclusion, platelets not only function as thrombocytes but they are key agents for tissue repair and organ regeneration. This is the first report that clarifies the precise mechanisms of platelet's proliferative effect on hepatocyte *in vitro*. In the near future, platelets could be used as a therapeutic strategy, i.e., infusion of the platelet rich fraction via the portal vein, for liver regeneration after an extended hepatectomy or other types of liver injury.

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## Figure Legends

**FIG.1.** Platelets significantly promote DNA synthesis in cultured hepatocytes.

(A) [<sup>3</sup>H]-methyl thymidine uptake by primary cultured hepatocytes was measured after incubation for 48 hours with or without platelets. Each column and bar represent the mean ±SD of 6 experiments. \* p<0.001 versus the platelet(-) group.

(B) Dose dependency of the proliferative effect of platelets. Platelets (50 µl) suspended at several cell densities (0/ml, 1x10<sup>5</sup>/ml, 1x10<sup>6</sup>/ml, 1x10<sup>7</sup>/ml, 2x10<sup>7</sup>/ml, and 1x10<sup>8</sup>/ml) were added to cultured hepatocytes, and hepatocyte BrdU uptake was measured after incubation for 48 hours. Each column and bar represent the mean ± SD of 8 experiments. \* p<0.001 versus the platelet(-)

10 group.

**FIG.2.** Direct contact was essential for hepatocyte proliferation. Hepatocytes and platelets were co-cultured with or without cell culture inserts that separated platelets from hepatocytes by a permeable membrane (pore size, 0.3 µm). Hepatocyte DNA synthesis was measured by [<sup>3</sup>H]-methyl thymidine uptake. Each column and bar represent the mean ± SD of 6 experiments. \* p<0.001 versus the platelet(-) group. † n.s. versus the platelet(-) group.

**FIG.3.** The platelet membrane did not exert a proliferative effect on hepatocytes. Platelet membrane, soluble fraction, and whole disrupted platelets were added to cultured hepatocytes, and [<sup>3</sup>H]-methyl-thymidine uptake was measured after incubation for 48 hours. Each column and bar represent the mean ± SD of 6 experiments. \* p<0.0001 versus the platelet(-) group. † n.s. versus the platelet(-) group.

**FIG.4.** Co-culture systems to elucidate the characteristics of direct contact. (A) Platelet(-) group: neither hepatocytes nor platelets were seeded in the upper chamber. (B) Co-mix group: platelets and hepatocytes were seeded in the lower chamber. (C) Separated co-culture group (Co-sep): platelets were seeded in the upper chamber. (D) Upper mix group: hepatocytes were seeded in the upper chamber and overlaid with platelets. (E) Thrombin stimulated group: platelets in the upper chamber were stimulated with 1 u/ml of murine thrombin to release soluble

factors such as cytokines and growth factors. (F) In each group, TLR2 cells in the lower chamber were counted after 72 hours of incubation. Each column and bar represent the mean  $\pm$  SD of 6 experiments. \*  $p < 0.001$  versus platelet(-) group

**FIG.5.** Platelets promote hepatocyte proliferation via the Akt and ERK1/2 pathways. Signal transduction of hepatocytes was analyzed after the addition of platelets. Hepatocytes were harvested at 1, 2, 5, 10, 20, 30, and 60 min after the addition of platelets. Activation of the Akt, ERK1/2, and STAT3 pathways was examined by Western blotting. The Akt pathway was activated within 1 min and ERK1/2 pathway was activated within 10 min after the addition of platelets; the STAT3 pathway was not. These results indicate that the mechanism by which platelets induce hepatocytes proliferation occurs via the activation of the Akt and ERK1/2 pathways, which are known to be related to growth factors.

**FIG.6.** Gel exclusion chromatography of platelet extracts. (A) The platelet extracts were gel filtrated on Superdex G200 gel. A solid line shows the resulting absorbance profile at 280 nm. The broken line shows the mitogenic activity of each fraction. Fractions 1 and 2 were nonspecifically macro-aggregated proteins. Significant mitogenic activity was observed in fractions 1, 2, 5-7, and 14-17. On Western blotting, fractions 4-6 were rich in HGF, fractions 5-7 were rich in VEGF, fractions 7-9 were rich in PDGF, and fractions 14-17 were rich in IGF-1. Fractions 9 and 10 were rich in hemoglobin contaminated in platelet collection. Values are given as x-fold stimulation over untreated controls. Data are means  $\pm$  SD of 8 experiments. (B) Cellular signals of hepatocytes stimulated with platelet growth factors. HGF, IGF-1, and VEGF activated the Akt and ERK1/2 pathways, which are involved in hepatocyte proliferation. It has been reported that platelets contain a large amount of PDGF; however, cellular signals were not activated by PDGF. Serotonin did not activate hepatocyte cellular signals with or without pretreatment with EGF (10 ng/ml).