Format for ANSWERING REVIEWERS

May 26, 2013

Dear Editor,

Title: Human platelets inhibit liver fibrosis in severe combined immunodeficiency mice

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The manuscript has been improved according to the suggestions of reviewers:
1 Format has been updated

2 Revision has been made according to the suggestions of the reviewer
Reviewer-1

(1) Platelets themselves are an important source of HGF. Hence, it is unclear why the authors speculate a role for Kupffer cells as the major producers of HGF. This needs to be explained considering that the authors demonstrate increased platelet concentrations in the liver.

→I appreciate reviewer's excellent indication. Actually, it is true that animal platelets (mouse and rats) contain large amount of HGF[1,2]. And, it is known that human platelets do not contain significant amount of HGF[3]. In this study, we demonstrated an immunostaining picture which showed large amounts of human platelets accumulating in the liver in the fibrotic liver (Figure 6). Furthermore, hepatic concentration of mice-specific HGF (measured by species-specific ELISA) was elevated in the hPLT group (Figure 4A). Therefore, we do not think mice-HGF was derived from human platelets accumulating in the liver. Moreover, since HGF is known to be predominantly produced by Kupffer cells in the liver[4], we first speculated that human platelets in the liver might have enhanced HGF release from Kupffer cells. However, since there is still possibility that human platelets induced mice-platelet accumulation, which can be another source of mice-HGF, we modified our first speculation and added this explanations in the forth paragraph of Discussion.

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2. **Matsuo R**, Ohkohchi N, Murata S, et al. Platelets strongly induce hepatocyte proliferation with IGF-1 and HGF in vitro. J Surg Res. 2008; **145**: 279. [PubMed: 17688880]
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4. **Knittel T**, Mehde M, Kobold D, Saile B, Dinter C, Ramadori G. Expression patterns of

matrix metalloproteinases and their inhibitors in parenchymal and non-parenchymal cells of rat liver: regulation by TNF-alpha and TGF-beta1. *J Hepatol* 1999; **30**: 48-60 [PMID:9927150]

(2) MMP-9 would result in increased inflammation and extracellular matrix turnover. Thus suggest that this may lead increased fibrosis. Also previous reports have in fact associated MMP-9 with liver fibrosis. Hence the author's findings needs explanation in this context since they note increased MMP levels with decreased fibrosis.

→ Thank you very much for indicating very important point. To the best of our research, there are two opposite view points regarding the role of MMP-9 in liver fibrosis, and it is controversial. It was reported that higher levels of MMP-9 was measured in HCV patients compared with healthy controls, and the serum MMP-9 concentration showed significant positive correlation with transaminase levels[1]. Moreover, hepatic expression of MMP-9 was significantly associated with increasing stage of fibrosis[2]. These studies were actually suggesting that MMP-9 would result in increased inflammation and extracellular matrix turnover, and eventually lead increased fibrosis. On the other hand, it was indicated that elevation in MMP-9 levels was not associated with the degree of fibrosis but linked to the histologically derived level of tissue inflammation[3]. Furthermore, in many previous studies, MMP-9 levels was used as one of indicators of liver fibrinolysis, and in fact, it was shown that induction of MMP-9 attenuated liver fibrosis[4-6]. Since we have been taking the latter view point, and studied MMP-9 as key enzymes responsible for the degradation of ECM [7], we think that induction of MMP-9 by human platelet transfusion helped attenuating liver fibrosis in this study.

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7. **Watanabe M**, Murata S, Hashimoto I, Nakano Y, Ikeda O, Aoyagi Y, Matsuo R, Fukunaga K, Yasue H, Ohkohchi N. Platelets contribute to the reduction of liver fibrosis in mice. *J Gastroenterol Hepatol* 2009; **24**: 78-89 [PMID:18624898 DOI: 10.1111/j.1440-1746.2008.05497.x]

Revieer-2

(3) The authors argued for an increase of hepatocyte apoptosis in treated mice but there are no statistical analyses for TUNEL staining, MMP-9 and Bcl-2 expression and the amount of cleaved caspase-3 might be compared to the non cleaved form. In these figures, the quality of western blot pictures needs to be improved.

→ Thank you very much for suggesting importance of quantification of each data. We have calculated TUNEL-positive hepatocytes/total hepatocytes and compared between the hPLT and PBS groups (Figure 4E). The index was significantly lower in the hPLT group than in the PBS group. We also refined our pictures, added non-cleaved caspase-3 and quantified MMP-9, Bcl-2, and cleaved caspase-3 expressions (Figure 4F,G). MMP-9 and Bcl-2 expressions were significantly higher in the hPLT group than those in the PBS group, whereas cleaved caspase-3/non cleaved-caspase-3 was lower in the hPLT group than in the PBS group (Figure 4G). We also quantified alpha-SMA expression and compared between the two groups (Figure 4E).

(4) The increase of p-MET and p-SMAD3 are convincing but the quantification of several independent experiments is required.

→We quantified phosphorylation of Met and SMAD-3 (Figure 5D). Phosphorylation of Met was significantly higher in the hPLT group than in the PBS group. Although the difference was not significant enough, phosphorylation of SMAD3 was lower in the hPLT group than in the PBS group ($P = 0.1$).

(5) The discussion is interesting but quite too speculative about the direct implication of Kupffer cells which are not formally characterized in this study.

→I appreciate reviewer's indication for our weakness in this study. We agree with the reviewer's comment. We did not demonstrate the direct proof regarding relationship between Kupffer cells and liver fibrosis in this study, and we made speculations by quoting references. Since HGF is known to be predominantly produced by Kupffer cells in the liver, we first speculated that human platelets in the liver might have enhanced HGF release from Kupffer cells. However, there was also the same comment from another reviewer and we thought that mice-platelets can become another source of HGF. Therefore, we changed our speculation into two possibilities, i.e., mice-platelet derived HGF and Kupffer cell derived HGF. We removed scheme (Figure 6 in the previous article) because it seems to be too strong and speculative to insist only from the present results. We added this explanation in the forth paragraph of Discussion.

(6) An important concern is the absence of significant difference in the sensitive markers of hepatic dysfunctions that include serum AST and ALT. How the authors explain changes in the fibrotic index without changes of these parameters?

→Thank you very much for indicating very important point. We also expected to have difference in both serum AST and ALT concentrations, however, there was only a tendency in the ALT concentration to be lower in the hPLT group than in the PBS group. In our fibrosis model using $CCl_4$ with these duration and dose (8 weeks, 200 μl/kg body weight), it was difficult to induce strong fibrosis and hepatocyte-apoptosis in SCID mice. Although there was a statistically difference in TUNEL-positive hepatocytes/total hepatocytes (Figure 4E), the difference was small, considering the damage of the whole liver (PBS group vs hPLT group; 5.9% vs 2.9% of total hepatocytes). We think this seemed to be one of reasons that we could not get the statistical difference in serum AST and ALT concentrations between the PBS and hPLT groups. We also changed Figure 4 C and D to more typical pictures which indicate actual average of apoptosis in both groups. We added this explanation in the last paragraph of Discussion.

3 References and typesetting were corrected

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology*.

Sincerely yours,

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