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Platelet aggregation but not activation and degranulation during the acute post-ischemic reperfusion phase in livers with no underlying disease

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Dear Dr. Heger,

Reviewers have submitted their critical appraisal of your paper. The reviewers' comments are appended below. Based on their comments and evaluation by the editorial board, your work was FOUND SUITABLE FOR PUBLICATION AFTER MINOR REVISION.

If you decide to revise the work, please itemize the reviewers' comments and provide a point-by-point response to every comment. An exemplary rebuttal letter can be found on at http://www.jctres.com/en/author-guidelines/ under "Manuscript preparation." Also, please use the track changes function in the original document so that the reviewers can easily verify your responses.

Your revision is due by Aug 11, 2015.

To submit a revision, go to http://jctres.edmgr.com/ and log in as an Author. You will see a menu item call Submission Needing Revision. You will find your submission record there.

Yours sincerely

Yao Liu
Editorial board member
Journal of Clinical and Translational Research

*****Reviewer comments*****

Reviewer #1: The authors describe some very intriguing observations when staining for platelets and P-selectin in warm liver ischemia reperfusion (I/R) injury. P-selectin staining of platelets is used as a marker for platelet activation. The observations are novel as the authors are the first to directly study platelet activation status in liver I/R. Unexpectedly, only minimal P-selectin staining of adherent platelets was observed 0-90 min after I/R injury. These observations contrast previous findings obtained using P-selectin knockout mice and anti-P-selectin blocking antibodies. In contrast to previous studies on the role of P-selectin in liver I/R injury, in the current study platelets, and also P-selectin, were directly labelled in vivo using infused anti-CD49b and anti-CD62P antibodies respectively, enabling visualisation of all platelets (and NK cells) and P-selectin expressing cells present. Moreover, the authors also stained neutrophils to determine the role of neutrophils in platelet adhesion to the liver microvasculature. The authors suggest that platelets adhere to the liver microvasculature in the absence of obvious platelet activation and minimal neutrophil involvement in platelet adhesion, a very interesting implication that has the potential to cause a shift in the current understanding of early liver I/R injury.

These are my comments:

The authors provide a table wherein the previous publications on the role of P-selectin, platelets and neutrophils are summarised. In my view the manuscript would greatly benefit from a somewhat extended description of some of these studies and other studies, for instance the work on the involvement of P-selectin in endotoxemia induced liver injury by Jaeschke et al. Related to that it appears that ex vivo labelling of platelets or the use of CD62Pknockout fail to explain all divergent results obtained in the previous studies, could the authors please comment (and please correct me if I am wrong!).

As the authors point out themselves, the time window after ischemia in which reperfusion is studied is very relevant, as at later time points neutrophil influx into the liver may be enhanced and this may be linked to platelet adhesion and potentially also activation. It would be useful to add the time window that was used monitor liver reperfusion in the previously published studies in the table. Notably, it has been suggested that cell death of liver sinusoidal endothelial cells (LSECs) is maximal after 90 min, the maximal time point that was also used in the present study. If LSEC cell death is mediated by platelet/neutrophil interactions this appears the correct study window.

The authors provide a very detailed description of the methods used for data analysis which is very useful for data interpretation, especially the results presented in Fig1E and background correction performed.

The authors write that no P-selectin staining of the liver microvasculature was observed. The authors need to point out in their manuscript that it has been described that LSECs are devoid of Weibel-Palade bodies and as such do not have an intracellular store of P-selectin and VWF available for release upon activation, but do express ICAM-1.

LSECs do not produce a basal lamina and it seems unlikely that collagen and laminin exposure are involved in platelet adherence in the this I/R model, it would be elegant to point out that by using anti-CD49b antibodies potential interactions of platelets with collagen or laminin may be blocked.

It is not immediately clear what distinguishes series A-D from F-H and I-L in Figure 1, also upon study of the figure legend. It seems that series A-D are derived from staining of platelets without P-selectin staining? That would form an important control; as using P-selectin antibodies one possibly blocks P-selectin interactions with leukocytes which upon adhesion may mediate further platelet adhesion. Moreover, P-selectin also binds to glycosaminoglycan (GAG) side chain of proteoglycans (chondroitin sulphate). Thus it would be imperative to show that platelet adhesion in this model is the same in the absence and presence of CD62Pantibodies. Alternatively, have the authors tried perfusing anti-CD62P antibodies after the start of liver injury?

Is it possible that ligand binding to P-selectin competes with the binding of P-selectin antibodies, could the authors please comment? Does the antibody bind with high affinity?

The puncture induced thrombosis model is perhaps not the most elegant model to confirm P-selectin staining, but suffices. One needs to bear in mind that the endothelial cells at the site of puncture do express P-selectin/may shed soluble P-selectin. Moreover, potential differences in platelet aggregate formation (assessed with CD49b staining) in the presence/absence of anti-CD62P antibodies were not assessed.

The shedding of soluble P-selectin (by platelets) can not be studied using the methods used herein. However, a role for soluble P-selectin in for instance atherosclerosis has been demonstrate. Can the authors please comment on a possible role of shedded, soluble P-selectin in Iiver I/R injury?

Supplemental movies were not available for review.

Authors' rebuttal:

Rebuttal - JCTRes-D-15-00003

The authors describe some very intriguing observations when staining for platelets and P-selectin in warm liver ischemia reperfusion (I/R) injury. P-selectin staining of platelets is used as a marker for platelet activation. The observations are novel as the authors are the first to directly study platelet activation status in liver I/R. Unexpectedly, only minimal P-selectin staining of adherent platelets was observed 0-90 min after I/R injury. These observations contrast previous findings obtained using P-selectin knockout mice and anti-P-selectin blocking antibodies. In contrast to previous studies on the role of P-selectin in liver I/R injury, in the current study platelets, and also P-selectin, were directly labelled in vivo using infused anti-CD49b and anti-CD62P antibodies respectively, enabling visualisation of all platelets (and NK cells) and P-selectin expressing cells present. Moreover, the authors also stained neutrophils to determine the role of neutrophils in platelet adhesion to the liver microvasculature. The authors suggest that platelets adhere to the liver microvasculature in the absence of obvious platelet activation and minimal neutrophil involvement in platelet adhesion, a very interesting implication that has the potential to cause a shift in the current understanding of early liver I/R injury.

Dear reviewer,

Thank you for your critical review of the manuscript and your comments/suggestions, which have resulted in a significantly improved paper. We have attempted to answer all your questions and implement the suggestions to the fullest possible extent, as indicated point-by-point below.

These are my comments:

[1a] The authors provide a table wherein the previous publications on the role of P-selectin, platelets and neutrophils are summarised. In my view the manuscript would greatly benefit from a somewhat extended description of some of these studies and other studies, for instance the work on the involvement of P-selectin in endotoxemia induced liver injury by Jaeschke et al.

Reply:

In order to rationalize why we investigated platelet CD62P expression in hepatic I/R injury, we summarized all previous platelet-focused liver I/R studies in Table 1, thereby exposing several inconsistencies in current literature. To allow a more easy interpretation of the presented results, a brief description of the used animal model(s) was added for each study. We are, however, not keen on expanding the table with non-I/R work as as we feel this takes away from the main purpose of this table. Because we do agree that the findings in the abovementioned study from Jaeschke's group are relevant for our manuscript, the suggested paper was referenced on page 16, line 18.

[1b] Related to that it appears that ex vivo labelling of platelets or the use of CD62Pknockout fail to explain all divergent results obtained in the previous studies, could the authors please comment (and please correct me if I am wrong!).

Reply:

The main rationale for performing the current study is that some of the previous reports on the role of platelets and/or CD62P in hepatic I/R injury are not fully supported by the experimental approach(es) used to arrive at these conclusions. In addition, we feel that direct *in vivo* labeling holds several advantages over *ex vivo* platelet staining and platelet reinfusion, which can induce handling artifacts and renders only a small fraction of platelets fluorescent. These considerations were mainly used to design our experiments and we do not claim that these factors account for all discrepancies shown in Table 1. Elaborating on the specific differences between these studies, however, is beyond the scope of the current (preliminary) work. As highlighted in the conclusion, follow-up experiments are required to elucidate the biological implications of our findings, which in turn would help to better understand the diverging conclusions of the studies presented in Table 1.

[2] As the authors point out themselves, the time window after ischemia in which reperfusion is studied is very relevant, as at later time points neutrophil influx into the liver may be enhanced and this may be linked to platelet adhesion and potentially also activation. It would be useful to add the time window that was used monitor liver reperfusion in the previously published studies in the table. Notably, it has been suggested that cell death of liver sinusoidal endothelial cells (LSECs) is maximal after 90 min, the maximal time point that was also used in the present study. If LSEC cell death is mediated by platelet/neutrophil interactions this appears the correct study window.

Reply:

The temporal nature of hepatic I/R injury should indeed be kept in mind when designing and performing hepatic I/R experiments. In line with the reviewer's suggestion, the studied reperfusion period per reference has been added to Table 1 (also see the reply to comment 1a). With respect to the reperfusion phase monitored in our work, rat liver I/R experiments from Clavien's group revealed that platelets predominantly induce sinusoidal endothelial cell apoptosis during the first hour of reperfusion, while exerting minimal effects on hepatocytes (*Gastroenterology 2000;118(1):183-91).* As argued by the reviewer, this makes the 90-min reperfusion period a suitable time frame for assessing platelet activation status in post-ischemic livers.

[3] The authors provide a very detailed description of the methods used for data analysis which is

very useful for data interpretation, especially the results presented in Fig1E and background correction performed.

Reply: n/a

[4] The authors write that no P-selectin staining of the liver microvasculature was observed. The authors need to point out in their manuscript that it has been described that LSECs are devoid of Weibel-Palade bodies and as such do not have an intracellular store of P-selectin and VWF available for release upon activation, but do express ICAM-1.

Reply:

We thank the reviewer for highlighting this (potential) phenotypical difference between sinusoidal and postsinusoidal endothelium. Accordingly, the following statement was added to the discussion:

Page 17, lines 1-5 now read: "*…and/or the fact that sinusoidal endothelium mainly relies on ICAM-1 instead of P-selectin to immobilize chemoattracted leukocytes following hepatic I/R. The latter may also relate to the reported absence of P-selectin-containing Weibel-Palade bodies in sinusoidal endothelial cells, albeit contradictory findings on this subject have been published.*"

However, we would like to note that post-sinusoidal venules do express CD62P under various sterile inflammatory conditions. As we simultaneously imaged the sinusoidal as well as post-sinusoidal vascular component, we feel that the statement on page 16 regarding the observed lack of endothelial CD62P expression remains valid.

[5] LSECs do not produce a basal lamina and it seems unlikely that collagen and laminin exposure are involved in platelet adherence in the this I/R model, it would be elegant to point out that by using anti-CD49b antibodies potential interactions of platelets with collagen or laminin may be blocked.

Reply:

In accord with the reviewer's comment, page 15, lines 16-17 now read (changes in bold):

"***Although the use of anti-CD49b antibodies deters a potential interaction of platelets with certain substrates (e.g., collagen),*** *this staining method has a high labeling efficiency, does not affect platelet phenotype [23], and obviates the need for intricate ex vivo platelet staining procedures that may affect platelet phenotype or function [27].*"

[6a] It is not immediately clear what distinguishes series A-D from F-H and I-L in Figure 1, also upon study of the figure legend. It seems that series A-D are derived from staining of platelets without P-selectin staining? That would form an important control; as using P-selectin antibodies one possibly blocks P-selectin interactions with leukocytes which upon adhesion may mediate further platelet adhesion.

Reply:

Panel A-D indeed show images of mice that only received fluorescent anti-CD49b and anti-CD31 antibodies (i.e., no anti-CD62P antibodies). We fully concur that it is crucial to better specify the used staining protocols in the figure legend to avoid confusion about the shown results. The Figure 1 legend has therefore been modified as follows (changes in bold):

*"Figure 1. Intravital imaging of platelet aggregation and platelet activation status following hepatic I/R in mice. (**A-D**) Platelet aggregates (red (**CD49b**), arrows) in hepatic microcirculation (blue, **CD31**) as a function of reperfusion time (left bottom, all imaging panels). Representative panels are shown per time point, taken from the video footage of 3 animals. (**E**) The mean pixel intensity per fluorescence channel (y-axis) was quantitated for each time point (x-axis) for every experimental group (resting platelets following I/R (CD49b + I/R), resting platelets in sham-operated animals (CD49b - I/R), and activated platelets following I/R (CD62P + I/R)) using FIJI/ImageJ software. Platelet fluorescence (flu) was normalized to endothelial fluorescence (mean ± SEM, sample size is given in parentheses in the legend). (**F-H**) Absence of P-selectin staining (green, **CD62P**) in post-ischemic liver microcirculation (blue, **CD31**). Incidental P-selectin-positive foci are indicated with arrows, corresponding to the same location at different reperfusion times. (**I-L**) Absence of platelet (red, **CD49b**) and neutrophil (green, **Gr1**) colocalization in post-ischemic hepatic microcirculation (blue, **CD31**). The quadrant corresponds to the same location at different reperfusion times, whereas the time lapse series in I-L correspond to panel C. Note the gradual increase in platelet aggregation in the demarcated region in this animal."*

[6b] Moreover, P-selectin also binds to glycosaminoglycan (GAG) side chain of proteoglycans (chondroitin sulphate). Thus it would be imperative to show that platelet adhesion in this model is the same in the absence and presence of CD62P antibodies. Alternatively, have the authors tried perfusing anti-CD62P antibodies after the start of liver injury?

Although not included in the main manuscript, pilot experiments revealed a similar extent of platelet aggregation in mice administered both anti-CD62P and anti-CD49b antibodies, thereby

excluding a potential influence of anti-CD62P therapy on the platelet plugs observed in Figure 1A-D. In the same pilot phase, several mice pretreated with anti-CD62P antibodies were given a second antibody bolus during reperfusion in attempt to establish the optimal antibody dose. As this approach failed to generate a detectable signal, we next switched to the hindleg thrombosis model shown in Figure S2 to validate our anti-CD62P antibody. Given the positive results generated in this model, we are confident that the absence of CD62P staining during the reperfusion phase is not related our choice of antibody or the timing of antibody administration.

[7]. Is it possible that ligand binding to P-selectin competes with the binding of P-selectin antibodies, could the authors please comment? Does the antibody bind with high affinity?

Reply:

Although not experimentally confirmed in the current work, we doubt that our results are confounded by a low binding affinity of the used anti-CD62P antibody. First, if a low antibody binding affinity would account for the complete lack of intrahepatic CD62P staining in the I/R experiments, in spite of the extensive platelet plugs shown in Figure 1A-D, we would expect that the hindleg thrombus model would also fail to produce a detectable signal. Second, we exclusively used the antibody portfolio of the Kubes lab for our experiments, which has been routinely used to monitor platelet activation with intravital microscopy in various thrombogenic and inflammatory settings (*e.g., Blood. 2005 Oct 1;106(7):2417-23*).

[8] The puncture induced thrombosis model is perhaps not the most elegant model to confirm P-selectin staining, but suffices. One needs to bear in mind that the endothelial cells at the site of puncture do express P-selectin/may shed soluble P-selectin. Moreover, potential differences in platelet aggregate formation (assessed with CD49b staining) in the presence/absence of anti-CD62P antibodies were not assessed.

Reply:

We readily acknowledge that P-selectin can be expressed and shed/released by both platelets and endothelium during thrombosis. The hindleg thrombosis model, however, only served to validate that our antibody indeed stained CD62P. For that purpose, it is not crucial to make the distinction between platelet, endothelial, or even soluble CD62P. Based on extensive previous experience with intravital imaging of activated platelets during thrombosis (see, e.g., *J Dermatol Sci 2011;63(3):139-47*), we feel that the observed changes in thrombus shape over time (i.e., embolization) indicate the antibody indeed stained platelet CD62P.

[9] The shedding of soluble P-selectin (by platelets) can not be studied using the methods used herein. However, a role for soluble P-selectin in for instance atherosclerosis has been demonstrated. Can the authors please comment on a possible role of shedded, soluble P-selectin in Iiver I/R injury?

Reply:

Shedding of P-selectin is part of the platelet activation cascade and thus can be used as a surrogate marker for platelet activation and thrombosis. Epidemiologic studies have repeatedly suggested that a correlation exists between systemic P-selectin levels and adverse outcomes in patients with cardiovascular disease (e.g., *J Am Coll Cardiol. 2014 Nov 4;64(18):1917-25, Atherosclerosis. 2015 Apr;239(2):405-11*). As there is currently no consensus on the involvement of secondary hemostasis in the pathophysiology of liver I/R injury, it seems unlikely that soluble P-selectin could serve a similar prognostic role in a surgical setting. One could also speculate that soluble P-selectin would actually attenuate hepatic I/R injury by serving as a soluble decoy ligand for leukocyte P-selectin glycoprotein ligand 1 (PSGL1). This possibility is plausible in light of the therapeutic efficacy of blocking interactions between P-selectin and leukocyte PSGL1 in experimental liver transplantation models (*J Immunol. 2006 Jan 1;176(1):616-24, Am J Transplant. 2013 Feb;13(2):299-311*).

Last, we would like to emphasize that follow-up experiments are warranted to clarify the biological implications of our findings. A first step would be to explore the relation between platelet aggregation in absence of P-selectin expression on the one hand and thrombosis, innate immune activation, and hepatocyte injury on the other hand. In particular, the association between early platelet aggregation and the release of P-selective positive platelet-derived microparticles (*PLoS One 2014;9(9):e104376, PLoS One 2014;9(9):e104376)* should be investigated as potential link between platelet plugging and post-ischemic liver injury.

[10] Supplemental movies were not available for review.

Reply:

All references to supplemental movies have been removed from the manuscript. The movies will be used in a manuscript reporting on a parallel study performed in the Kubes lab.

2nd editorial decision:

Date: 11-Sep-2015

Ref.: Ms. No. JCTRes-D-15-00003R1
Platelet aggregation but not activation and degranulation during the acute post-ischemic reperfusion phase in livers with no underlying disease
Journal of Clinical and Translational Research

Dear Dr. Heger,

I am pleased to inform you that your manuscript has been accepted for publication in the Journal of Clinical and Translational Research.

Comments from the editor and reviewers can be found below.

Thank you for submitting your work to JCTR.

Kindest regards,

Yao Liu
Editorial Board Member
Journal of Clinical and Translational Research

Comments from the editors and reviewers:

Reviewer #1: Thank you for the detailed response and the changes to the manuscript, all my comments have been addressed.