ANSWERING REVIEWER 1

Mar 3, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 8716-review.doc).

Title: Osteopontin (OPN) is induced in human alcoholic liver disease, in vivo and in vitro by acute alcohol exposure and mediates direct alcohol effects on hepatic stellate cell gene expression and function

Author: Devanshi Seth, Alastair M Duly, Paul C Kuo, Geoffrey W McCaughan, Paul S Haber.
Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 8716

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

ANSWERING REVIEWER 1

(1) Correct spellings:
pg 2, Abbreviations: Quantitative
pg 5, Abstract: assays
pg 9, Cell Culture: immunohistochemistry
pg 12, last para: "In vitro LX2 cells exposed to acute alcohol"

(2) Please shortly explain the rationale to analyze the OPN-C isoform.
The following has been added: pg 7, 1st para.
Transcriptional isoform OPN-C is known to be associated with more aggressive tumors and poor prognosis for cancers (Refs 12, 13), but its role remains controversial (Ref 14). We were the first to observe that alcohol predominantly induced SPP1-C subtype in advanced ALD and HSCs (Ref 15), indicating the importance of studying its role in alcoholic liver injury. Please also see modified para 1 on pg 16.

(3) Please explain abbreviations like INR in legend of table 1.
Inserted: Table 1, pg 30
INR: International Normalised Ratio for prothrombin time to predict survival

(4) "Protein expression (WB) confirmed mRNA increase." Please correct this sentence for clarity.
Corrected, pg 12, 3rd para.
Protein expression (WB) corroborated Q-PCR results for mRNA increase.

(5) Figure 4 Images and quantitative data for 2, 6 and 7 do not fit, please check.
Explanation: Images in the panel show only the migrated side of the membrane. For integrity and consistency, images shown represent all treatments only from a single experiment. The graph, depicting the ratio of migrated cells over non-migrated cells, is representative of data derived from at least 3 experimental replicates and 6 fields per treatment.

(6) Discussion, please explain OPN-A is only mentioned in the legend of figure 1, please clarify.
The following has been inserted/modified for clarification: pg 12, last para.
Expression of both total full length OPN-A and isoform OPN-C isoform mRNAs were tested in LX2 cells ± alcohol. OPN-A and OPN-C mRNAs significantly increased by 15-fold and 286-fold, respectively, within 4h of 10 mM alcohol exposure compared to untreated control (Fig 1E).
Please also see modified para 1 on pg 16.

ANSWERING REVIEWER 2

(1) Why alcohol effect was investigated in hepatic stellate cells (HSC) and not in hepatocytes? Especially when using an inhibitor of ethanol metabolization (4-MP) when it is well known that hepatocytes are the major ethanol-metabilizing cell type in the liver. Explain and discuss why HSC were investigated at all and what is known already about alcohol and HSC (do they express ADH at all?) and about OPN in HSC.
Text modified and reference added, pg 17, para 2: **HSCs are shown to metabolise alcohol via alcohol dehydrogenase (Ref 39).**

Added information explaining the choice of using HSC cell line LX2, pg 16, para 1.
Explanation: Hepatocyte cell lines Huh7 and HepG2 were studied with respect to OPN. Our preliminary results showed differential expression of the isoforms in hepatocytes and hepatic stellate cells in response to alcohol in our cell culture models. The greatest up-regulation of OPN-C expression was seen in advanced ALD and in LX2 cells compared to hepatocyte cell lines HepG2 and Huh7. This is now mentioned and referenced (Refs 15, 32). Activated HSCs are known to produce OPN (Refs 33-35).

(3) The western blot shown in figure 3A and its' quantification is not very convincing. All changes are below a 2-fold difference to control and there is no dose-dependent effect.
Graphs in 3A are corrected/revised. Text changed accordingly in Results, pgs13, para 2. The graphs show moderate but significant changes. These are representative of 3 experimental replicates and 4-5 animals per treatment group, however, the images shown are only from 3 animals from a single experiment for consistency. Quantitation was done as ratio of each phosphorylated band to its corresponding total expression and calculated as fold-change from control per treatment group.

(4) How many animals per condition were used for Figure 3D?
Animals used: N=4 per group for WT (dose response experiments) and 6-8 per group (knockout experiments). Figure legends' text modified for better clarification, pg 26, para 1.

(5) TGFbeta goes down by alcohol but aSMA increases.
This finding was unexpected, but our observations consistently and significantly showed TGFb was downregulated in vivo with alcohol. More importantly, in the primary TGFb producing stellate cells TGFb was significantly upregulated in LX2 with alcohol. Text inserted on pg 17, para 3.

(6) KO-mouse without alcohol is a missing control which is needed to see if any effect is really due to the alcohol treatment or only due to the knockout of OPN.
Figure (Suppl Fig 2) showing comparison between WT and OPN-/- gene expression is added. Text inserted pg 14, para 1.

(7) Why is there a basal OPN expression in the KO which is almost as high as in the untreated wild-type?
The basal expression of OPN mRNA is significantly inhibited by more than 3-log fold in knockouts compared to WT. Importantly, OPN protein was not detected in OPN-/- mice with or without alcohol treatment, even when PCR amplified residual OPN mRNA (Suppl Fig 1, text inserted pg 14, para 1). Suppl Fig 1 is added to the manuscript in support of this.

(8) Fig. 3E aSMA should be shown as well.

Increased aSMA expression (flow cytometery and immunofluorescence) in LX2 cells ± alcohol was published by us (Ref 23) and is now referred in the text on pg 17, para 3.

(9) "SPP1" is mentioned - what is that?

SPP1 (Secreted phosphotprotein 1) is another name for OPN. SPP1 is replaced by OPN. Pg 16, line 1.

(10) On the same page is discussed how CD44 upregulation in LX-2 cells might contribute to metastasis formation in HCC. This line of discussion does not seem to make much sense - HCC cells are malignant hepatocytes, not HSCs. Also, this study is about acute effects of alcohol, cancer formation is clearly a consequence of chronic damage.

For clarity and focus, this line of discussion is removed from the manuscript.

We thank the reviewers for their invaluable comments and advice.

3 References and typesetting were corrected

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

Sincerely yours,

Peter Laszlo LAKATOS, MD, PhD
1st Dept. of Medicine
Semmelweis University
Budapest, Koranyi 2A
H-1083-Hungary
Fax: +36-1-313-0250
E-mail: kislakpet@bell.sote.hu