Author's response to reviews

Title: Co-culture of primary human tumor hepatocytes from patients with hepatocellular carcinoma with autologous peripheral blood mononuclear cells: Study of their in vitro immunological interactions.

Authors:

Polyxeni P Doumba (jdoumba@yahoo.gr)
Marilena Nikolopoulou (nik.milena@yahoo.com)
Ilias P Gomatos (gomatosilias@yahoo.com)
Manousos M Konstadoulakis (konstadoulakismm@yahoo.com)
John Koskinas (koskinasj@yahoo.gr)

Version: 2 Date: 25 September 2012

Author's response to reviews:

Athens 25.09.12
To the editor,

We submit the revised manuscript entitled “Co-culture of primary human tumor hepatocytes from patients with hepatocellular carcinoma with autologous peripheral blood mononuclear cells: Study of their in vitro immunological interactions.”

We believe that the manuscript has been improved and contains all the comments/revisions raised by the reviewers.

The content of the manuscript has not been published or submitted elsewhere.

This work has been accepted as a poster in the International Liver Congress™ 2009 - 44th annual meeting of the European Association for the Study of the Liver April 22 - 26, 2009 - Copenhagen, Denmark.

All authors have contributed significantly and agree with the content of the manuscript.

Furthermore no conflict of interest applies to all authors.

We enclose a full point by point description of the changes made and our answers to reviewers’ comments.

The changes in the text are illustrated by bold letters.

We also want to thank the reviewers for their valuable comments.

Thank you for considering the revised manuscript for publication in your journal.

John Koskinas MD, PhD
Associate Professor of Internal Medicine and Hepatology
Academic Department of Internal Medicine, Medical School of Athens
Hippokration General Hospital, Athens

Point-by-point description
Answers to comments
Reviewer 1

Comment 1: Although I acknowledge the technical value of the culture of primary parenchymal human liver cells, I believe the work is preliminary at present. The FACS plots are not convincing at all, and the MHC-II expression of the “hepatocytes” indicates that there might be “contamination” by other cells. The authors only mention negative staining for CD14 or CD3, but this should be shown. The quality of the presented FACS data is rather poor (likely due to the low numbers of cells retrieved from human tissue), but this makes the data difficult to interpret. I think it is important to show that (a) no cell doublets were measured (FSC-W), and (b) all “hepatocytes” stain negative for CD45 (leukocytes), endothelial and HSC markers. Furthermore, immunohistochemistry for typical KC and HSC markers (e.g., CD68, decorin) should be performed.

Answer to comment 1
• The isolation protocol of hepatocytes (tumor, non-tumor and normal) used in these experiments was modified from other published isolation protocols and the appropriate changes were done in order to be appropriate for the specific liver specimens that we used. For example, the triple centrifugation at 81 g for 12 min at 40°C was performed in order to remove the other liver cell populations (non-parenchymal cells). After each centrifugation both the supernatant (that contained the non-parenchymal cells) and the pellet (that contained the hepatocytes) were seen under the microscope in order to estimate the purity of hepatocytes. We concluded that the pellet indeed contained the purified hepatocytes, as was further confirmed by flow cytometric analysis of the two hepatocyte specific markers Hep-Par 1 and albumin and light microscopy. CD14 negative staining also verified the purity of hepatocytes. The figure with the CD14 flow cytometric staining has been added to the Results Section (Figure 3b). Also part of this answer has been added in the Methods section “The pellet containing the liver cells was re-suspended in ice-cold DMEM-F12 with 20%FBS and triple centrifugation at 81 g for 12 min at 40°C was performed in order to separate the purified hepatocyte population (pellet) from the non-parenchymal cells (supernatant) (20)”.

• To further purify the hepatocyte population we performed a pre-culture step in order to remove any remaining non-parenchymal cells such as Kupffer and stellate cells. Hepatocytes were plated in 60-mm dishes without collagen I and remained in culture for 4h-10h. This further purified our hepatocyte population because Kupffer and stellate cells need less time in culture to adhere compared to hepatocytes. Then, we carefully removed the hepatocytes and proceeded as described in the method section for the purified population. Part of this answer has been added in the Methods section “…as further confirmed by light microscopy and by flow cytometric analysis of the two hepatocyte specific
markers Hep-Par 1 and albumin described in the next paragraphs. Next, hepatocytes remained in culture for few hours in 60-mm dishes without collagen I (a pre-culture step) in order to achieve maximum hepatocyte purity, by removing possible remaining nonparenchymal cells. Then, we carefully removed the supernatant containing the hepatocytes…"

• Moreover, the adhered hepatocytes were washed twice in order to remove any remaining immune cells (they do not adhere). CD3 negative staining also confirmed the absence of lymphocytes. In addition, by performing the above washes all immune cells were removed and this was also confirmed by light microscopy. Therefore, no CD45 staining was needed. The figure with the CD3 flow cytometric staining has been added to the Results Section (Figure 3a). Part of this answer has been added in the Methods section “16 hours prior to co-culture experiment the hepatocyte medium was removed and hepatocytes were washed twice with 1x PBS in order to remove the possible remaining intrahepatic lymphocytes”.

• Furthermore, the flow cytometric quantrium analysis in Figure 4a, reveals that the only cells that express MHC class II molecules are the hepatocytes, (M2 region shows double staining of albumin and MHC class II molecules). M4 region in the quantrium flow cytometric diagram depicts that no other liver cells express MHC-II.

• After the trypsinization step, hepatocytes were seen under the microscope in order to verify if all hepatocytes were detached from the plate and that no hepatocyte clusters were formed. If hepatocytes clusters were formed then we further treated hepatocytes with StemPro® Accutase® Cell Dissociation Reagent (Life technologies, Gibco, USA), similar to trypsin, that detaches the cells and dissolves the clusters. Therefore, before flow cytometric analysis of hepatocytes we have verified that no cells doublets were present. Part of this answer has been added in the Methods section “In addition, hepatocytes were treated with Stem Pro Accutase (Life technologies, Gibco, USA) in order to avoid the formation of cell clusters”.

• Hepatocytes and PBMCs were analyzed in different flow cytometric protocols, because due to their different size and granulation, the settings varied between these two cell types.

Comment 2: The co-culture experiments are, to my opinion, difficult to interpret. The co-staining of CD8 and HLA-DR is not convincing, because the compensation appears not appropriate. How should CD8+ cells and hepatocytes/HCC cells “communicate”? Why do both populations upregulate MHC-II? I strongly doubt that these observations are truly relevant. More functional assays would be needed to confirm that these observations are not artefacts, but related to pathogenically relevant mechanisms.

Answer to comment 2

• HLADR (MHC class II) marker is a marker of T cells activation, including CD8+ T cells (reference 24 in the paper). HLA-DR expression on hepatocytes as well as on other APCs is an indicator that these cells can act as antigen presenting cells. It is well known that under certain circumstances (ie. viral injury and
cancer) hepatocytes act as antigen presenting cells and that MHC II expression increases in the presence of IFN-gamma (references 15, 34,35 in the paper).

- MHC-II expressing hepatocytes (HCC and non-HCC) act as antigen presenting cells to CD4+ T cells, and then CD4+ T cells can activate CD8+ T cells and possibly initiating an antitumor immune response.
- The compensation was set before starting the experiments for both CD8-FITC and HLADR-PE monoclonal antibodies.

Comment 3: Co-cultures of hepatocytes / tumor cells with defined immune cell subpopulations (e.g., isolated by MACS) are needed to identify and delineate the interactions

Answer to comment 3
- Our target was firstly to investigate if hepatocytes (tumor and non-tumor) could express MHC class II molecules and act as antigen presenting cells and secondly to study the immune interaction between hepatocytes and PBMCs. We chose to analyze the total PBMCs population because this was closer to the in vivo system, as a more realistic approach of this interaction. Moreover, we also chose to study specifically the CD8+ T cells because they are the effector T cell subset and their activation may initiate an effective immune response.
- These experiments have a double aim. First to establish the human co-culture system and secondly to investigate possible interactions with the immune peripheral cells. Of course further experiments as well as more functional tests should perform in order to delineate the interaction between the MHC-II expressing tumour hepatocytes and immune cells and how this interaction could lead in an effective antitumor immune response. These experiments were further beyond the scope of this study.

Answers to comments
Reviewer 2

Minor comment: The shown graphs do not depict standard deviation (SD) as described in methods passage. The authors should check this issue and complete their graphical illustration with this information.

Answer to comment:
- All the graphs in the paper depict the standard deviation (SD), The changed figures are Figure 4c,d, Figure 5c and Figure 6b.