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Prediction of drug-induced immune-mediated hepatotoxicity using hepatocyte-like cells derived from human embryonic stem cells

Dong Eon Kim[a,b,1], Mi-Jin Jang[c,1], Young Ran Kim[c,d,1], Joo-Young Lee[c], Eun Byul Cho[a], Eunha Kim[c], Yeji Kim[c], Mi Young Kim[c], Won-il Jeong[a,b], Seyun Kim[c,**], Yong-Mahn Han[c,**], Seung-Hyo Lee[a,b,*]

[a] Biomedical Science and Engineering Interdisciplinary Program, Daejeon, 34141, South Korea
[b] Graduate School of Medical Science and Engineering, Biomedical Research Center, Daejeon, 34141, South Korea
[c] Department of Biological Sciences, KAIST Institute for the BioCentury, Korea Advanced Institute of Science and Technology (KAIST), Daejeon, 34141, South Korea
[d] Division of Life Science, Korea Basic Science Institute, Daejeon, 34133, South Korea

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ABSTRACT

Drug-induced liver injury (DILI) is a leading cause of liver disease and a key safety factor during drug development. In addition to the initiation events of drug-specific hepatotoxicity, dysregulated immune responses have been proposed as major pathological events of DILI. Thus, there is a need for a reliable cell culture model with which to assess drug-induced immune reactions to predict hepatotoxicity for drug development. To this end, stem cell-derived hepatocytes have shown great potentials. Here we report that hepatocyte-like cells derived from human embryonic stem cells (hES-HLCs) can be used to evaluate drug-induced hepatotoxic immunological events. Treatment with acetaminophen significantly elevated the levels of inflammatory cytokines by hES-HLCs. Moreover, three human immune cell lines, Jurkat, THP-1, and NK92MI, were activated when cultured in conditioned medium obtained from acetaminophen-treated hES-HLCs. To further validate, we tested thiazolidinedione (TZD) class, antidiabetic drugs, including troglitazone withdrawn from the market because of severe idiosyncratic drug hepatotoxicity. We found that TZD drug treatment to hES-HLCs resulted in the production of pro-inflammatory cytokines and eventually associated immune cell activation. In summary, our study demonstrates for the first time the potential of hES-HLCs as an *in vitro* model system for assessment of drug-induced as well as immune-mediated hepatotoxicity.

1. Introduction

Acute drug-induced liver injury (DILI) is a significant cause of morbidity and a major hurdle during drug development. The rate of liver failure caused by drug toxicity is about 13% in clinical trials during new drug development (Uetrecht, 2009a). Over 800 medical treatments currently in use are known to induce hepatotoxicity (Leise et al., 2014). Furthermore, 4% of all new medical entities are withdrawn from the market due to adverse drug reactions (ADR) in the liver (Edling et al., 2008). DILI is, thus, the most significant cause of the cessation of drug development during preclinical stages, the unanticipated termination of clinical tests, and the withdrawal of approved drugs from the market (Leise et al., 2014).

Drugs or their reactive metabolites can have marked effects on gene expression, function, and cellular homeostasis in hepatocytes (Yildirimman et al., 2011). Although many of these drug actions can directly lead to hepatotoxic responses and cell death, there is emerging evidence supporting the hypothesis that immune cells play a critical role in drug-induced hepatotoxicity. The direct toxicological effects of drugs on liver cells may be an initiating event for an immune response, which determines the extent of liver damage (Uetrecht 2009a; Uetrecht 2009a; 2009b). Furthermore, the recruitment and activation of immune cells such as lymphocytes and macrophages may increase hepatic inflammation, and the intrahepatic localization of lymphocytes determines the progression and severity of liver injury (Uetrecht 2009b; Navarro and Senior 2006; Edling et al., 2009). This drug-induced

Abbreviations: DILI, drug induced liver injury; hES-HLCs, hepatocyte-like cells derived from human embryonic stem cells; APAP, acetaminophen; TZD, thiazolidinedione; TNF, tumor necrosis factor; IL, interleukin; IFN, interferon; PHHs, primary human hepatocytes; hES-DE, definitive endoderm cells induced from hESCs

* Corresponding author at: Cellular Immunology Laboratory, Graduate School of Medical Science and Engineering, KAIST, 291 Daehak-ro, Yuseong-gu, Daejeon, 34141, South Korea.
** Corresponding authors at: Department of Biological Sciences, KAIST, 291 Daehak-ro, Yuseong-gu, Daejeon, 34141, Korea.
E-mail addresses: seyunkim@kaist.ac.kr (S. Kim), ymhan57@kaist.ac.kr (Y.-M. Han), sl131345@kaist.ac.kr (S.-H. Lee).
[1] These authors made equal contributions to this work.

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immune-mediated hepatotoxicity primarily involves the action of immune cells mediating inflammatory processes through the release of several pro-inflammatory cytokines and chemokines including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, interferon (IFN)-α, CCL5, and CXCL8, which attract and activate other leukocytes and lymphocytes (Uetrecht, 2009a).

Primary human hepatocytes (PHHs) and animal cell cultures are currently used as the most physiologically relevant models for examining drug-induced hepatocyte toxicity *in vitro*. However, the predictive nature of these models is severely limited because of the scarcity and poor quality control of these cells. In addition, interspecies differences in drug responsiveness can exist, and toxicity testing using animals on a large scale raises ethical and economic concerns.

The efficient differentiation of pluripotent stem cells offers a novel approach to generate an unlimited supply of human hepatocytes (Kim et al., 2011). Using our knowledge of pharmacology, stem cell biology, and immunology, we have developed a highly stable and sensitive model with the potential to assess drug-induced immune-mediated hepatotoxicity. As a proof of concept, we evaluated the hepatotoxic responses of hepatocyte-like cells derived from human embryonic stem cells (hES-HLCs) when treated with acetaminophen (APAP), a classic example of a hepatotoxic drug. In particular, we wanted to assess the potential for use of drug-treated hES-HLC culture medium for immune-based toxicity screening. In order to assess whether APAP-induced liver injury (AILI) could affect the responses of immune cells, we developed an *in vitro* co-culture system utilizing three different immune cell lines including Jurkat T-lymphocytes, THP-1 monocytes, and NK92MI natural killer (NK) cells (Supporting information Fig. 1). Finally, we chose to validate our model with the thiazolidinedione (TZD) class of anti-diabetic drugs since the medical use of these drugs has been discontinued due to their severe idiosyncratic hepatotoxicity. We detected the release of pro-inflammatory cytokines from TZD-treated hepatocytes and associated activation of immune cells *in vitro*. In summary, we show that the hES-HLCs-based assay offers a reliable platform to quantitatively assess drug-induced, immune-mediated hepatotoxicity with statistical significance. This setup and the associated methods are thus suitable for screening hepatotoxic drugs with the potential for causing immune activation.

2. Materials and methods

2.1. Differentiation of hESCs to hepatocyte-like cells

Undifferentiated hESCs (CHA-hESC4) were maintained using a feeder-free system with hESC medium, as previously described (Rho et al., 2006). CHA-hESC4 line was established and obtained from CHA hospital, Seoul, Republic of Korea. Differentiation of hESCs into hepatocyte-like cells (HLCs) was as previously described (Cai et al., 2007). Briefly, for the differentiation of hESCs into definitive endoderm (DE), the cells were cultured in RPMI-1640 (Hyclone, Logan, UT) including activin A (50 ng/ml, Peprotech, Rocky Hill, NJ) for 5 d. For the differentiation of hESC-DE cells into early HLCs, the cells were cultured in the presence of FGF4 (30 ng/ml, Peprotech) and BMP2 (20 ng/ml, Peprotech) for 5 d. These early HLCs were cultured with HGF (20 ng/ml, Peprotech) for 5 ds. For maturation into hepatocytes, the cells were cultured in the presence of oncostatin M (10 ng/ml, R & D System, Minneapolis, MN) and dexamethasone (0.1 μM, Sigma-Aldrich, St Louis, MO). This study was approved by the 'Institutional Review Board (IRB)' at Korea Advanced Institute of Science and Technology (KAIST, KH2012-29) and all experimental methods were performed in accordance with the relevant guidelines and regulations by KAIST.

2.2. PHHs culture

PHHs (Invitrogen, Lot no. Hu4246, Waltham, MA) were thawed and plated according to the supplier's protocol. Briefly, cells were thawed at 37 °C for 30–60 s followed by dilution into 50 ml of warmed CHRM® Medium (Cryopreserved Hepatocyte Recovery Medium, BD biosciences, San Diego, CA). The cell suspension was centrifuged at 100 × *g* for 10 min. Cells were plated in Medium (CHRM®) containing the Hepatocyte plating supplement pack (BD biosciences) on 12 well collagen-coated tissue culture plated (Corning, Corning, NY) at a seeding density of 5×10^5 cells/well. After 6 h, the medium was replaced with incubation medium containing CHRM® medium and Hepatocyte Maintenance supplement pack. PHH cultures were maintained in incubation medium for 24 h, followed by incubation with DMEM-low glucose medium (1000 mg/l, WelGENE, Daegu, Korea) for 2 h.

2.3. Reverse transcription-PCR (RT-PCR)

Total RNA was isolated from CHA-hESC15 cells and hESC-DE cells using easy-BLUE (Intron Biotechnology, Seoul, Korea), and cDNAs were synthesized from RNAs using M-MLV Reverse Transcriptase (Enzynomics, Daejeon, Korea), according to the manufacturer's instructions, respectively. RT-PCR was performed with Taq Plus DNA polymerase (BioAssay, Hayward, CA) on a GeneAmp® PCR System 9700 (Life Technologies, Carlsbad, CA). The following conditions were used for RT-PCR: 95 °C denaturation for 5 min, 25–35 cycles of 60 °C annealing for 30 s, and 72 °C elongation for 5 min. Transcription level of *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) gene was used for normalization. Quantitative RT-PCR (qRT-PCR) was performed on a CFX-Connect Real-Time System (Bio-Rad, Hercules, CA) with a premade 2× mixture. The 2× mixture consisted of 40 mM Tris pH 8.4, 0.1 M KCl, 6 mM $MgCl_2$, 2 mM dNTP, 0.2% fluorescein, 0.4% SYBR Green, and 10% DMSO. For comparative analysis, the expression level of *GAPDH* was used for normalization. Information of the primer sets used in this study is described in Supporting information Table 1.

2.4. Fluorescence-activated cell sorting (FACS) analysis

hESCs and hES-DE cells were incubated with Accutase (Innovative Cell Technologies, San Diego, CA) for 10 min at 37 °C for harvest in single cell-dissociated form. In the case of hES-HLCs, cells were incubated with 10 mg/ml of collagenase type I (Thermo Fischer Scientific, Waltham, MA) for 15 min at 37 °C before treatment of Accutase. Dissociated cells were washed with phosphate-buffered saline (PBS) containing 1% FBS, followed by 30 min of incubation with allophycocyanin (APC) mouse anti-human CD 184 (CXCR4), CD45, APC mouse IgG2a k Isotype, or FITC IgG1 k Isotype (BD biosciences) at 4 °C. For intracellular FACS of albumin, cells were permeabilized with Fixation/Permeabilization solution (BD Biosciences) for 1 h at room temperature (RT). After permeabilization, cells were incubated with rabbit anti-albumin antibody (DAKOCytomation, Glostrup, Denmark) followed by 1 h of incubation with Alexa 488-conjugated secondary antibody (Invitrogen, Waltham, MA) at RT. All the samples were analyzed using FACS Calibur (BD biosciences), and evaluation of positive population was performed using FlowJo software (Tree star, Ashland, OR).

2.5. Immunofluorescence analysis

Cells were cultured in 24-well culture plates (SPL life sciences, Seoul, Korea) for immunocytochemistry. Cells were fixed with 4% formaldehyde (Sigma-Aldrich) at RT, permeabilized with 0.1% Triton X-100 for 15 min at RT, and blocked with 3% donkey serum (Jackson ImmunoResearch, West Grove, PA) for 1 h at RT. Primary antibodies targeting a-1-antitrypsin (DAKOCytomation) and albumin (DakoCytomation) were diluted with 1:200 in blocking solution and incubated with the cells overnight at 4 °C. After several subsequent washes with PBS-0.1% Tween 20 (PBST), cells were incubated with the fluorescent-conjugated secondary antibody (1:200, Alexa Fluor 488 or 594; Invitrogen,) for 1 h at RT. Finally, cells were washed with PBST