Japanese Journal of Gastroenterology and Hepatology

Research Article

A Hepatocelluar Co-Culture System for the Investigation of Cellular-Based Mechanisms of Troglitazone-Induced Liver Injury

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Received: 28 May 2020

Accepted: 10 June 2020

Published: 15 June 2020

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1. Abstract

1.1. Objective: Drug-induced liver injury (DILI) is a serious issue faced by the pharmaceutical industry, and often leads to drug withdrawal once patients experience drug-induced severe hepatic failure. We developed an *in vitro* assay system that enables to assess cellular damage and immune reaction responses that ultimately result in DILI using troglitazone as a model drug.

1.2. Methods: A co-culture system consisting of a human liver cancer cell line, HuH-7, and the human promyelocytic leukemia cell line, HL-60, was developed for the evaluation of troglitazone-induced changes ingene expression related to chemoattractant and cellular stress. HL-60 cells were subjected to differentiation by 12-*O*-Tetradecanoylphorbol 13-acetate (TPA) to be a macrophage-like lineage before co-cultures.

1.3. Results: In our co-culture system, more HL-60 cells attached to HuH-7 cells in a TPA-differentiation- and troglitazone-dependent manner. Our system consisting of liver cancer cells and promyelocytic leukemia cells has clarified the importance of genes encoding interleukin (IL)-8 and heat shock protein (Hsp) 70.

1.4. Conclusions: Our co-culture system will be useful for identification of factors associated with various hepatotoxic drugs and for screening for possible DILI-inducingagents.

2. Keywords: Drug-induced liver injury; Troglitazone; Human hepatocellular carcinoma; Human promyelocytic leukemia; Differentiation; Phorbol ester; Macrophage-like cells; Immune system; Co-culture; Inflammatory cytokines

3. Introduction

Drug-Induced Liver Injury (DILI) is one of the most serious issues in the pharmaceutical industry and often leads to drug withdrawal after patients experience drug-induced severe hepatic failure [1].Therapeutic drugs that are known to induce DILI include antibiotics, anti-diabetic agents, anti-arrhythmic agents, anti-rheumatoid agents, anti-pneumonia agents, angiotensin receptor blockers, anti-hyperlipidemia agents, anti-convulsants, and non-steroidal anti-inflammatory drugs. DILI has an estimated annual incidence between 10 and 15 per 10,000 to 100,000 persons exposed to prescription medications [2].

DILI has been classified as intoxicated DILI and idiosyncratic DILI. The former is thought to occur by the administered drug itself or by its metabolites produced through the bio activation process catalyzed by drug/xenobiotic metabolizing enzymes (DMEs) as in the case of acetaminophen [2]. It often occurs in a dose-dependent fashion. Conversely, idiosyncratic DILI are further classified into one due to allergic idiosyncrasy and the other due to rare DME properties where patients possess generally rare polymorphic alleles. Id-

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iosyncratic DILI is generally triggered by the modification of cellular proteins by reactive intermediates formed by catalytic activities of DMEs.

Antibodies against presumably drug-protein adducts, drug-DME protein adducts, and so-called Liver Kidney Microsomal (LKM) antibodies [3, 4], have been detected in DILI patients to drugs such as carbamazepine [5], diclofenac [6, 7], tienilic acid [8], dihydralazine [9], and halothane [10].

These results indicate that drugs/xenobiotics that produce drug/xenobiotic-protein adducts or drug/xenobiotic-DME adducts require bio activation, and that subsequent immunological reactions occur for the onset of DILI. Thus, *in vitro* assays, which enable to evaluate both the metabolic activation process and immune reaction process, but ultimately result in DILI, are needed.

In the present study, we created a co-culture system consisting of a human liver cancer cell line, HuH-7, and the human promyelocytic leukemia cell line, HL-60, for the evaluation of biological events through cell-cell interaction between liver cells and immune/inflammatory cells using troglitazone as a model drug. HL-60 cells underwent differentiation by 12-*O*-tetradecanoylphorbol 13-acetate (TPA) to a macrophage-like lineage.

4. Materials and Methods

4.1 Chemicals

Troglitazone was purchased from Wako Co. Ltd. (Wako Pure Chemical Industries, Ltd., Osaka, and Cat. No. 203-17603); 12-*O*-Tetradecanoylphorbol 13-acetate (TPA) was from Wako Co. Ltd. (Wako Pure Chemical Industries, Ltd., Osaka, Cat. No. 166-23594); and all other chemicals used in the present study were of the highest grade available.

4.2. Cell and Culture Conditions

Human liver cancer cells, HuH-7, were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°Cin a humidified atmosphere of 5% CO_2 and 95% air. Human promyelocytic leukemia cells, HL-60, were cultured in RPMI1640 medium supplemented with 10% FBS at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. For co-culturing of HuH-7 and HL-60 cells, DMEM supplemented with 10% FBS and RPMI1640 supplemented with 10% FBS were mixed 1:1 (co-culture medium) and co-cultured in 6-well Nunc Cell Culture Inserts with pore size of 0.4 micron (Thermo Fisher Scientific, Rosklide, Denmark, and Cat. No.140660). HuH-7 cells were inoculated onto the bottom of the well and HL-60 cells were inoculated in the inserts. Before co-culture, both cells were maintained for at least one week in the co-culture medium.

4.3. Differentiation of HL-60 by 12-O-Tetradecanoylphorbol 13-Acetate (TPA)

HL-60 cells were treated with 16 nM TPA in the co-culture medium at a density of approximately 1-5×10^5 cells/mL to induce cells to terminal differentiation [11]. We measured nitroblue tetrazolium (NBT, Sigma-Aldrich, N5514) reduction activity as a marker of cell differentiation [12] to identify the macrophage-like lineage by exposure of the cells to 400 mg/mL NBT after harvesting the cells at 4 hr, 24 hr, and 48 hr. NBT reducing activity began to increase at 4 hr and reached plateau at 48 hr (Data not shown).

4.4 Treatment with Troglitazone of the Co-Cultured Huh-7 and HL-60 Cells

The co-cultured cells were layered in Nunc Cell Culture Inserts (Cat. No. 140660, Nunc, Rochester, NY, USA) were exposed to 50 μM troglitazone at 37℃ for 48 hr. Both HuH-7 and HL-60 cells were inoculated at cell densities of 4×10^5 cells/dish/3 mL of co-culture medium. HL-60 cells, which had been treated either with or without 16 nM TPA for 48 hr, were used for the co-culture experiments. To examine direct contact of HuH-7 and HL-60 cells (stained by DiO (Vybrant Cell-Labelling Solutions, Cat. No. V22885, Invitrogen, Eugene, OR, USA) in an 8-chamber wells equipped chamber slide (IWAKI Science Products Dept. 5722-008) with chamber volume of 1 mL, HL-60 cells (1×10^6 cells/mL) were treated with 16 nM TPA at 37°C for 48 hr. Then, 3×10^5 cells/mL HL-60 cells were stained with DiO (250-fold dilution) in RPMI1640 with 10% FBS overnight. HuH-7 cells (1×10^4/chamber/200 μL) were inoculated in a chamber slide. Three hours later, 100 μL of 1×10^4 of DiO-stained HL-60 cells or 100 μL of 1.5×10^4 of TPA-differentiated and DiO-stained HL-60 cells were inoculated on HuH-7 cells. Two hours later, treatment of the co-culture cells began with either vehicle (dimethyl sulfoxide) or 50 μM troglitazone, and the co-cultured cells were cultured for 2–3 days. The HL-60 cell suspension was removed from the chamber and was washed extensively with DMEM with neither FBS nor phenol red. Green fluorescent was detected if DiO-stained HL-60 cells were attached with HuH-7 cells by excitation at 484 nm and emission at 501 nm by fluorescent microscopy (IX81, Olympus, and Tokyo, Japan).

4.5 Gene Expression Analyses

HuH-7 cells were lysed by RLT buffer (RNeasy Mini kit, QIAGEN, Hilden, Germany). Likewise, HL-60 cells were similarly lysed after being harvested from the culture insert. Cell lysis was performed according to manufacturer's instructions. CDNAs were prepared by oligo(dT) and random hexamers using Transcriptor First Strand cDNA Synthesis Kit (Roche, Cat. No. 04 897 030 001, Mannheim, Germany). Transcripts (β-actin [ACTB], Hsp70, and IL-8) were quantified by real-time

polymerase chain reaction (RT-PCR) (7500 Real Time PCR Systems, Applied Biosystems, Foster City, CA 94404), using the following primers sequences:

ACTB Forward 5'-ACGGCCAGGTCATCACTATTG-3'

ACTB Reverse 5'-ATACCCAAGAAGGAAGGCTGGA- 3

Hsp70 Forward 5'-GGCAAGGAGCTGAACAAGAG- 3

Hsp70 Reverse 5'-GATAGTGGCGTTCCTCTGGA-3

IL-8 Forward 5'-ATGACTTCCAAGCTGGCCGT- 3

IL-8 Reverse 5'-ACAGAGCTCTCTTCCATCAGA-3

5. Results

5.1. Attachment of Differentiated Macrophage-Like HL-60 Cells to Huh-7 Cells in the Co-Culture System Treated with Troglitazone

As the immune system has been shown to play an important role in DILI, we created a co-culture system involving liver cells and immune cells. HL-60 cells that were differentiated by phorbol ester such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or untreated were utilized as immune cells. Terminal-differentiated and macrophage-like HL-60 cells by TPA treatment were co-cultured with HuH-7 cells in an 8-chamber equipped chamber slide and treated with 50 μM troglitazone for 48 hr. As shown in (Figure 1), in the presence of TPA-differentiated HL-60, the morphology of HuH-7 cells differed in comparison to non-differentiated HL-60 cells. Although qualitative, differentiated HL-60 cells were able to attach directly to HuH-7 cells, which was in contrast to non-differentiated HL-60 cells co-cultured with HuH-7 cells or with cells treated with and without 50 μM troglitazone. These results prompted us to examine changes in gene expressions for the chemo attractant IL-8 and cellular stress-responsive Hsp70.

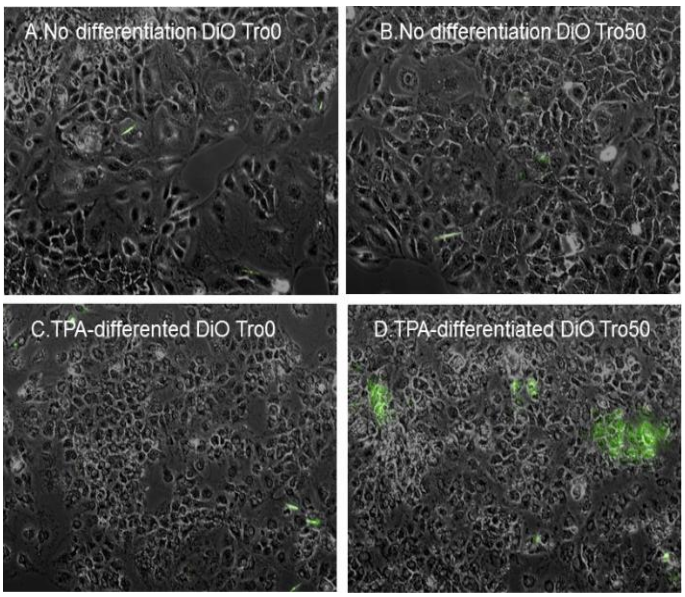

Figure 1: Attachment of HL-60 cells to HuH-7 cells in a co-culture treated with 50 mM troglitazone

5.2. Changes in Gene Expression Encoding Chemo attractants and Proteins Associated with Cellular Stress in Differentiated Macrophage-Like HL-60 Cells and Huh-7 Cells in the Co-Culture System

We evaluated changes in gene expression related to cellular stress and chemo attractants using the co-cultured HuH-7 cells and HL-60, which had undergone terminal differentiation by 16 nM TPA.

As illustrated in (Figure 2), both HuH-7 and differentiated HL-60 cells showed a tendency for the highest IL-8 expression under the condition of co-culture in a 6-well Nunc Cell Culture Insert exposed to 50 μM troglitazone (Co-culture TPA-diff. HL-60 50 μM Tro in (Figure 2). This tendency was most strongly manifested in differentiated HL-60 cells. (Figure 3) illustrates Hsp70 expression in each of HuH-7 and HL-60 cell-lines, which were co-cultured and treated with or without 50 μM troglitazone together with mono-culture of each cell-line treated with or without 50 μM troglitazone. As for the results in IL-8, both HuH-7 and differentiated HL-60 cells showed a tendency for the highest Hsp70 expression under the co-culture condition exposed to 50 μM troglitazone (Co-culture TPA-diff. HL-60 50 μM Tro in (Figure 3). The results shown in (Figures 2 and 3) suggest that HL-60 cells differentiated to be macrophage-like cells and HuH-7 cells interacted with each other through humoral factors, and both cells showed strong responsiveness to troglitazone exposure. (Figure 3) also shows a remarkable increase in the Hsp70 expression was observed in the TPA-differentiated HL-60 which interacted with HuH-7 cells. Results on IL-8 were consistent with the phenomena of cell attachment shown in (Figure 1). Overall, our experiments suggested troglitazone treatment of liver cancer cells, HuH-7, which interacted with terminal-differentiated HL-60 cells through some humoral factors, activated cytokine production including chemo attractant IL-8 and stress-responsive gene, Hsp70, in both HuH-7 and terminal-differentiated HL-60 cells.

Figure 2. IL-8 expression in HuH-7 and HL-60 cells relative to respective monoculture or co-culture with native or TPA-differentiated HL-60 treated with troglitazone

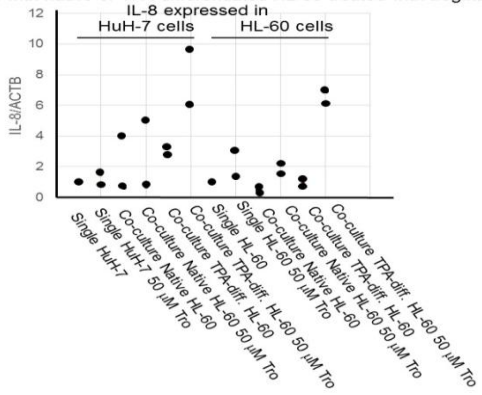

Figure 2: IL-8 expression in HuH-7 and HL-60 cells relative to respective single cultures and co-culture with native or TPA-differentiated HL-60 treated with troglitazone.

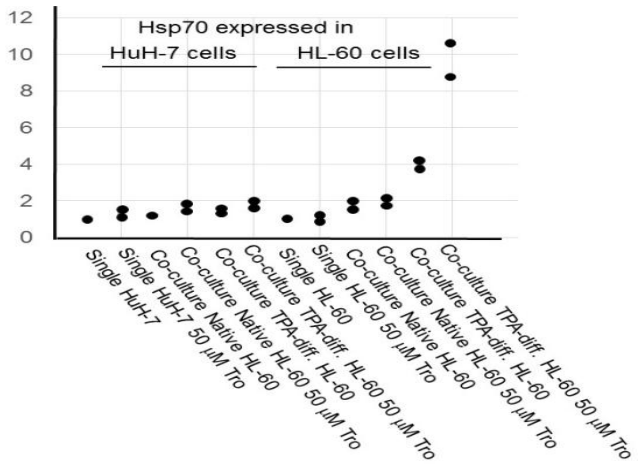

Figure 3: Hsp70 expression in HuH-7 and HL-60 cells relative to respective single culture and co-culture with native or TPA-differentiated HL-60 treated with troglitazone.

6. Discussion

In most cases of DILI-causing drugs, damaged cellular proteins produced by reactive intermediates are then recognized as non-self by immune surveillance mechanisms [13-18].

We co-cultured human liver cancer HuH-7 cells and human myelocytic leukemia HL-60 cells treated with TPA to induce macrophage-like differentiation. Next, co-cultured cells were exposed to 50 μM troglitazone to examine troglitazone-associated changes in gene expression in HuH-7 and HL-60 cells. Before the evaluation of gene expression, we set out to examine cell attachment of DiO-stained HL-60 cells, which were either TPA-differentiated or not, overlaid on HuH-7 cells in consideration of a review article on lymphocyte adhesion to hepatic sinusoid via cell adhesion molecules [19]. Although qualitative, TPA-differentiated HL-60 cells treated with troglitazone easily attached to the HuH-7 cell layer. The results of HL-60 cell attachment after treatment of the co-culture system with troglitazone prompted us to examine changes in gene expression associated with chemo attractant (IL-8) and cellular stress response (Hsp70). Real-time PCR revealed that IL-8 expressions were elevated in the highest degree in both HuH-7 and HL-60 cells whose culture conditions were troglitazone-treated, and the presence of TPA-differentiated HL-60 cells. In the case of Hsp70, the most remarkable degree of elevation of Hsp70 expression was observed in TPA-treated HL-60 cells, where the co-culture system was treated with 50 μM troglitazone. Besides IL-8 and Hsp70, DNA-damage inducible transcript 3 (DDIT3) and metallothionein 2A (MT2A) gene expression was also tested. In these cases, 50 μM troglitazone remarkably increased gene expression of both genes even in the HuH-7 cell monoculture, while no appreciable difference was observed with co-cultured HuH-7 cells (data not shown). These results may suggest that TPA-differentiated HL-60 cells co-cultured with HuH-7 cells communicated with each other in response to troglitazone to induce IL-8 and Hsp70 genes as compared with mono-cultured HuH-7 and HL-60 cells. The co-culture-dependent increase in gene expressions was limited to IL-8 and Hsp70, which might be an event specific to the interaction of liver cells with macrophage-like cells treated with troglitazone. Many other known DILI-causing drugs should be evaluated for their effects on gene expression changes at the transcriptional and translational levels. Our result on the importance of IL-8 was consistent with a previous report on the mechanistic insight of various DILI-inducing drugs by [20]. It should be noticed that no specific metabolically activating enzyme for troglitazone [21, 22] was included in the current co-culture system, as HuH-7 cells reportedly expressed higher CYP3A4-dependent activities than HepG2 cells [23, 24]. Nevertheless, plausible cellular reactions such as induction of IL-8 and Hsp70 expression occurred. Either a limited troglitazone activation might have occurred in the system, or unmetabolized troglitazone might have induced IL-8 and Hsp70 genes.

In conclusion, our co-culture system consisting of liver cancer cells and promyelocytic leukemia cells has clarified the importance of the inflammatory environment and cellular stress, namely IL-8 and HSP70 for mechanistic insights of troglitazone-induced liver injury. This system will be useful for identification of factors associated with various hepatotoxic drugs and for screening of possible DILI-inducing agents. With the accumulation of research findings on the causal mechanisms of DILI, *in vitro* systems to predict whether a new drug candidate may be considered a possible DILI-causing drug will continue to be essential.

HL-60 cells were stained with DiO as described in Materials and Methods. The stained HL-60 cells were overlaid on HuH-7 cells in a chamber slide. Both cells in the co-culture system were treated with 50 mM troglitazone for 2-3 days. HL-60 cells were removed from the chamber and washed extensively with DMEM (w/o FBS and phenol red). The attached HL-60 cells were detected by excitation at 484 nm and emission at 501 nm by fluorescent microscopy. A representative data set is shown. In the presence of TPA-differentiated HL-60, morphology of HuH-7 cells changed in comparison to those in the presence of non-differentiated HL-60 cells. Although a qualitative assessment, TPA-differentiated HL-60 cells treated with troglitazone easily attached to the HuH-7 cell layer.

The co-culture system was created as described in Materials and Methods. Each dot represents measurement of IL-8 mRNA level normalized by β-actin (ACTB) obtained by average of two measured values. "Co-culture Native HL-60" indicates HL-60 cells not subjected to differentiation by TPA were co-cultured with HuH-7 cells. "Co-culture Native HL-60 50 μM Tro" indicates HL-60 cells differentiated by 50 μM TPA treatment co-cultured with HuH-7 cells exposed to 50

μM TPA.

The co-culture system was created as described in Materials and Methods. Each dot represents a measurement of Hsp70 mRNA level normalized by β-actin (ACTB) obtained by the average of two measured values. "Co-culture Native HL-60" indicates HL-60 cells not subjected to differentiation by TPA were co-cultured with HuH-7 cells. "Co-culture Native HL-60 50 μM Tro" indicates HL-60 cells differentiated by 50 μM TPA treatment co-cultured with HuH-7 cells exposed to 50 μM TPA.

7. Conflict of Interest

This work was supported by Grants-in-Aid from the Ministry of Education Science and Culture Japan (Medical Innovation by Advanced Science and Technology: MIAST); and from the Human Science Foundation (KHD1222).

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