Evaluation of Three-Dimensional Cultured HepG2 Cells in a Nano Culture Plate System: an In Vitro Human Model of Acetaminophen Hepatotoxicity

Kohei Aritomi¹,² #, Yoichi Ishitsuka¹,² #,*, Yoshiro Tomishima¹, Daisuke Shimizu¹, Nazuki Abe¹, Tsuyoshi Shuto², Mitsuru Irikura¹, Hirofumi Kai², and Tetsumi Irie¹,³

1Department of Clinical Chemistry and Informatics, Graduate School of Pharmaceutical Sciences, 2Department of Molecular Medicine, Graduate School of Pharmaceutical Sciences, 3Center for Clinical Pharmaceutical Sciences, Faculty of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Chuo-ku, Kumamoto 862-0973, Japan

Received August 4, 2013; Accepted December 13, 2013

Abstract. Overdoses of acetaminophen (paracetamol, N-acetyl-p-aminophenol; APAP) cause severe liver injury, yet there is no common or high throughput in vitro human APAP model. This study examined the characteristics and usefulness of HepG2 cells grown in a nano culture plate (NCP) system, a three-dimensional culture method, as an in vitro human model for APAP-induced hepatotoxicity. The NCP-cultured HepG2 cells showed higher expression of mRNA and protein levels of cytochrome P450 2E1, which metabolizes APAP to a toxic metabolite, APAP-cysteine adduct formation, and higher sensitivity against APAP-induced cell injury compared with conventionally cultured cells. We demonstrated that treatment of APAP in NCP-cultured HepG2 cells shows key mechanistic features of APAP-induced hepatotoxicity, such as decreases in intracellular glutathione and mitochondrial membrane potential, activation of JNK, and cellular injury; and pharmacological agents, such as Cyclosporine A (a mitochondrial permeability transition inhibitor) and SP600125 (a JNK inhibitor), prevented cell injury induced by APAP exposure. In addition, the antidote of APAP-induced hepatotoxicity, N-acetylcysteine, could attenuate cellular injury induced by APAP in NCP-cultured HepG2 cells. We suggest that cellular injury induced by APAP treatment using an NCP-HepG2 system is a useful human model to study mechanisms and screen drug candidates of APAP-induced hepatotoxicity.

Keywords: acetaminophen, liver injury, HepG2 cell, three-dimensional culture, human model

Introduction

Acetaminophen (paracetamol, N-acetyl-p-aminophenol, APAP) is a widely used analgesic/antipyretic drug with proven safety at therapeutic doses (1). However, the hepatotoxicity induced by an APAP overdose in suicidal or unintentional ingestions is a critical issue, and it is the most frequent cause of acute liver failure in the United States (2), United Kingdom (3), and other countries (4, 5). Liver injury induced by APAP overdose is characterized by extensive hepatocellular necrosis, which is initiated by N-acetyl-p-benzoquinone imine (NAPQI), a reactive metabolite of APAP, that is mainly metabolized by cytochrome P450 (CYP) 2E1 (6, 7). Although some cellular events, such as glutathione (GSH) depletion, mitochondrial dysfunction, and the JNK pathway, may be involved in APAP-induced hepatotoxicity, the precise mechanisms of toxicity have not been identified (8 – 10).

In general, to evaluate pathophysiological mechanisms and screen new drugs against diseases, detailed in vivo and in vitro models of disease are essential. Most knowledge of APAP-induced liver injury has been obtained in studies on rodents. However, differences
in the pathogenesis of APAP-induced hepatotoxicity between rodents and humans appear to be considerable (11 – 13). To address the limitations of animal models of APAP-induced hepatotoxicity, a human in vitro system may be useful. HepG2 cells, a human hepatoma cell line, have been widely used as a model of hepatocytes because of its broad utility, stability, and ease of handling compared with primary hepatocytes. Unfortunately, fundamental liver functions, such as CYP enzyme expression and albumin production, generally deteriorate over time in HepG2 cells. Therefore, HepG2 cells have not been considered useful as an in vitro system for an APAP-induced hepatotoxicity model (14, 15).

Recent reports have demonstrated that the micro-space cell culture plate method, a three-dimensional (3D) culture system, can induce hepatocyte-specific functions, including CYP2E1 expression, in HepG2 cells, which results in an increase in cytotoxicity by acetaminophen compared with a conventional two-dimensional (2D) culture system (16). These results suggest that 3D-cultured HepG2 cells may be a useful tool as an in vitro human model of APAP-induced hepatotoxicity. Other studies have reported on a new 3D-cultured HepG2 system using a nano culture plate (NCP) that shows higher expression of albumin and some CYP enzymes in NCP-cultured HepG2 cells compared with conventionally cultured cells (17). However, it remains to be demonstrated whether cell injury induced by APAP in 3D-cultured HepG2 cells in vitro was produced by corresponding human and rodent mechanisms of in vivo APAP-induced hepatotoxicity, and if antidotes such as NAC or other agents also show protective potential against APAP-induced hepatotoxicity in the 3D-cultivation system.

This study was conducted to gain insight into the value of 3D-cultured HepG2 cells as a human system to study APAP-induced hepatotoxicity. We evaluated whether 3D-cultured HepG2 cells by the NCP method showed distinctive mechanistic characteristics, such as decreases in intracellular GSH and mitochondrial membrane potential and the phosphorylation of JNK during APAP-induced cell injury. In addition, we assessed the effects of NAC or other antidotes of APAP-induced hepatotoxicity on cellular injury induced by APAP in this system.

Materials and Methods

Chemicals and materials

NanoCulture® plates (NCPs; 96- or 24-well micro square pattern type, Catalog No. NCP-LS96 for 96-well and NCP-LS24 for 24-well plate) were purchased from SIVAX Co. (Kawasaki). Acetaminophen (> 99%), N-acetylcysteine (NAC), protease from Aspergillus oryzae, and sodium acetate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Cyclosporine A (CyA) and anthrax [1-9-cd] pyrazol-6 (2H)-one (SP600125) were purchased from LC Laboratories (Woburn, MA, USA). Acetaminophen-cysteine adduct (APAP-CYS) was obtained from Santa Cruz Biotechnology (Heidelberg, Germany). NANOSSEP® 30K OMEGA was purchased from Pall Corporation (Port Washington, NY, USA). Methanol of the highest grade was purchased from Wako Pure Chemical Industries, Ltd. (Osaka). Dimethyl sulfoxide, dehydrated (DMSO) was purchased from Wako Pure Chemical Industries, Ltd. The cell counting kit and Cellstain® Double Staining Kit were obtained from Dojindo Laboratories (Kumamoto). A High Capacity cDNA Reverse Transcription Kit and Fast SYBR® Green Master Mix were purchased from Applied Biosystems (Life Technologies Japan, Tokyo). An RNasea Plus Mini Kit® was purchased from Qiagen (Hilden, Germany). BIOXYTECH GSH/GSSG-412™ was purchased from OXIS Health Products, Inc. (Portland, OR, USA). Dulbecco’s modified Eagle’s medium containing a low glucose concentration (DMEM) was purchased from Gibco® Life Technologies Japan, Tokyo). HyClone™ fetal bovine serum (FBS) was purchased from Thermo Scientific (Logan, UT, USA). Other reagents and solvents were of reagent grade. Deionized and distilled water was used throughout the study.

Cell cultures and morphology

The human hepatoblastoma cell line HepG2 (RIKEN Bio Resource Center, Tokyo) was incubated at 37°C under an atmosphere of 95% air and 5% CO2 in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Confluent cultures were washed with phosphate-buffered saline (PBS), pH 7.4, detached with trypsin, centrifuged, and sub-cultured. In the conventionally cultured group, the HepG2 cells were seeded onto a Nunc™ 96- or 24-well culture plate (Thermo Scientific) at a density of 3 × 104 cells/well in 100 μL culture medium in 96-well plates or 1.5 × 105 cells/well in 500 μL culture medium in 24-well plates. In contrast, 96- or 24-well NCPs were used in the NCP-cultured group, and the cells were seeded at the same density compared with the conventionally cultured group. The cell morphologies were photographed and analyzed using a microscope system (BIOREVO BZ-9000; Keyence Co., Osaka).

Quantitative real-time PCR analysis

Quantitative reverse transcription polymerase chain reaction assays (qRT-PCR) were performed to measure the mRNA expression levels as previously described.
with minor modifications (18). Briefly, total RNA was extracted using an RNeasy Plus Mini Kit® according to the manufacturer’s instructions. The qRT-PCR for human cytochrome P450, family 2, subfamily E, polypeptide 1 (CYP2E1; NM_000773.3), and β-actin (NM_001101.3) was performed using the following primers: CYP2E1 sense, TTACGGTGTCACCCCTGTTTCCGCTGCTGTTGTCGCT-3'; β-actin sense, AGATGAGCCAGGCTGCTGCT and antisense; AACCCTCATTTGCAATGG. Total RNA was transcribed to cDNA using a High performance liquid chromatography with electrochemical detection system (HPLC-ECD) 

**Determination of APAP-cysteine adduct by high performance liquid chromatography with electrochemical detection (HPLC-ECD)**

We examined APAP-CYS adducts formation, a measure of NAPQI production (Supplementary Fig. 1: available in the online version only), in NCP-cultured and conventionally-cultured HepG2 cells. After protease digestion of the cellular protein, APAP-cysteine (APAP-CYS) adducts were measured in cells and in the culture medium by ECD-HPLC. The determination was performed by the methods previously described by McGill et al. (19) and Muldrew et al. (20) with minor modifications. In brief, HepG2 cells cultured in a 24-well conventional plate or NCP and exposed to 15 mM APAP for 24 h were detached and lysed in lysis buffer (25 mM Tris and HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) and stored at −30°C until analysis. A 100-μL aliquot of the sample was placed on a NANOSEP® centrifugal device with a membrane molecular weight cutoff of 30 kDa (previously conditioned by wetting with 200 μL of distilled water and drying by centrifugation at 12,000 × g for 10 min) and was centrifuged at 12,000 × g for 10 min. The protein retained on the membrane was washed three times with 100-μL portions of distilled water, with centrifugation at 12,000 × g for 10 min after each washing, and then quantitatively dislodged from the membrane with the aid of a spatula and 100 μL portions of distilled water until a 200 μL volume of protein dispersion had been collected. This protein dispersion was mixed, in succession, with 12.5 μL of protease solution (8 units/mL) and then incubated at 50°C for 16 h on a heating block. The digest was transferred to a conditioned NANOSEP® centrifugal device and centrifuged at 12,000 × g for 10 min to collect the filtrate. A 50-μL aliquot of this solution was injected into the liquid chromatography system. The linearity of the ECD-HPLC method was confirmed by preparing a calibration curve from serial dilutions (0.2, 0.5, 1, 2.5, 5, and 10 μg/mL) of the standard sample of APAP-CYS (Santa Cruz). ECD-HPLC analysis was performed at 25°C under isocratic conditions using PU-980 from JASCO Corp. (Tokyo) and a Coulochem II model 5200 detector from ESA (Bedford, MA, USA). An automated injection (25 μL) was performed using an 851-AS from JASCO Corp. The mobile phase was 50 mM sodium acetate and 7% methanol, pH 4.8. The flow rate was 1.0 mL/min through a reversed phase packed column (5C18-MS-II, 4.6 ID × 150 mm, COSMOSIL; Nacalai Tesque, Kyoto). The run time was 20 min. The peak detection limit was set to 1 μA. In this study, the optimal potential for analysis of APAP-CYS was 400 mV. APAP-CYS levels in the unknown samples were quantified using peak height of the channel and standard curves for APAP-CYS. APAP-CYS values in unknown samples were expressed as μg of APAP-CYS per mg protein by whole cell protein quantitative determination. The detection limit of APAP-CYS was approximately 0.8 ng/mg of protein in our assay conditions.

**Immunoblot analysis**

HepG2 cells cultured in a 24-well conventional plate or NCP were detached and lysed in lysis buffer [25 mM Tris HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and a 1% Halt™ protease inhibitor cocktail (Thermo Scientific)] and stored at −30°C until further analysis. Cell lysates were subjected to SDS-PAGE on a 10% polyacrylamide gel. Proteins were transferred onto a PVDF membrane (Millipore Corp., Bedford, MA, USA). After blocking with 5% skim milk, the membranes were washed three times and treated with the primary antibody. Primary antibodies were diluted in 5% skim milk at the following dilutions: anti-CYP2E1 antibodies (Abcam, Inc., Cambridge, MA, USA) (1:2500) or anti-β-actin (Cell Signaling Technology, Danvers, MA, USA) (1:1000), incubated overnight at 4°C. To determine the JNK signaling, Phospho-SAPK/JNK and SAPK/JNK rabbit Ab (Cell Signaling Technology) were used at a dilution of 1:1000 and incubated overnight at 4°C. Subsequently, the membranes were washed three times with TBS in Tween-20 and counterstained with the corresponding secondary antibodies conjugated to anti-rabbit IgG (1:3000). The membranes were visualized using Pierce enhanced chemiluminescence (ECL) western blotting reagents and ECL film.
Cell viability and cell death assays

APAP-induced viability of HepG2 cells was evaluated by a cell viability assay and by the calcein-acetomethoxy (calcein-AM) and propidium iodide (PI) (which stain viable and dead cells, respectively) dual-staining assay as previously described (21). Cell viability was measured using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, namely, the water-soluble tetrazolium salt (WST-8) assay, using a Cell Counting Kit (Dojindo Laboratories) according to the manufacturer’s protocol. HepG2 cells were seeded at 3 × 10⁴ cells/well into a 96-well conventional plate or NCP for 48 h before APAP exposure. The cells were treated with APAP (0 – 20 mM) for 3 to 48 h at 37°C and 5% CO₂. The cells were then incubated with 10 μL of WST-8 reagents that were added to each well followed by incubation for 3 h and measured at an absorbance of 450 nm using a micro plate reader (Tecan Co., Ltd., Männedorf, Switzerland). Cell viability was expressed as a percentage of the viable cells relative to the untreated controls. Moreover, cell death was observed by measuring the fluorescence of calcein-AM and PI at the excitation/emission wavelengths of 490/510 nm and 530/580 nm, respectively, using a fluorescent microscope (Biorevo, Keyence Co.). NAC (1 – 100 μM) was dissolved in DMEM, and CyA and SP600125 were dissolved in DMSO (final concentrations of DMSO were always < 0.1%, which has been shown to have no effects on cell viability). To evaluate the effects of these pharmacological reagents against APAP-induced cell injury, HepG2 cells cultured in a conventional plate or NCP were exposed to 15 mM APAP in the presence or absence of the reagents at various concentrations. Cell viability was measured 48 h after APAP exposure.

Measurement of intracellular glutathione, phosphorylation of JNK, and mitochondrial membrane potential

The intracellular GSH concentration was measured in accordance with methods described previously in our laboratory with minor modifications (18). HepG2 cells were seeded at 3 × 10⁴ cells/well into a 96-well conventional plate or NCP for 48 h before APAP exposure. The cells were exposed to APAP (15 mM) for 0 – 48 h at 37°C and 5% CO₂. Subsequently, the cells were detached from the plates and aliquoted into test tubes. After centrifugation and removal of the supernatant, 300 μL of 5% metaphosphoric acid (Wako Pure Chemical Industries) aqueous solution was added to the samples that were then vortexed for 20 – 30 s. Glutathione concentrations were determined using BIOXYTECH GSH/GSSG-412 according to the manufacturer’s instructions. Changes in intracellular GSH levels were expressed as a percentage of the untreated controls.

The activation of the JNK pathway was assessed by the measurement of phosphorylation of JNK. HepG2 cells were seeded at 1.5 × 10⁵ cells/well into a 24-well conventional plate or NCP for 48 h before APAP exposure. Cells were treated with 15 mM APAP for 0 – 12 h at 37°C and 5% CO₂, and the cells were collected at each time point. As described above, JNK and phosphorylated JNK were determined by immunoblot analysis.

Mitochondrial membrane potential was determined with the JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA). In intact cells, the dye preferentially localizes to mitochondria, where it forms aggregates that fluoresce red. When the mitochondria membrane potential decreases, that is membrane permeability transition (MPT), with cellular injury, the dye transports to the cytosol and fluoresces green. Therefore, the ratio of red-to-green fluorescence reflects mitochondrial membrane integrity. HepG2 cells were seeded at 3 × 10⁴ cells/well into a 96-well conventional plate or NCP for 48 h before the APAP exposure. The cells were exposed to APAP (15 mM) for 0 – 48 h at 37°C and 5% CO₂. Subsequently, the cells were stained with JC-1 fluorescence reagent according to the manufacturer’s instructions. The cells were photographed and analyzed using a microscope system (BIOREVO BZ-9000) and quantitatively determined using Image J (http://rsb.info.nih.gov/ij/). Total mitochondrial membrane potential in treated cells was expressed as a percentage of the fluorescence ratio compared with the untreated controls.

Statistical analyses

Results were expressed as the mean ± S.D. Statistical analyses were performed using GraphPad Prism ver. 5.01 (GraphPad Software, San Diego, CA, USA). Multiple comparisons were made to examine the statistical significance of the data. When uniform variance was identified by Bartlett’s analysis (P > 0.05), one-way analysis of variance (ANOVA) was used to test for statistical significance. When significant differences (P < 0.05) were identified, the data were further analyzed using the Tukey (or Tukey–Kramer) multiple range test.

Results

Morphological and functional differences and variation of cell viability following exposure to APAP in conventionally and NCP-cultured HepG2 cells

As shown in Fig. 1A, conventionally-cultured HepG2 cells adhered to the bottom of the plate, whereas NCP-cultured HepG2 cells were aggregated and became spheroids. The mRNA expression level of CYP2E1 in
the NCP-cultured group was 8-fold greater than that of the conventionally cultured group (Fig. 1B). In addition, protein levels of CYP2E1 were significantly increased in the NCP-cultured group compared with that of the conventionally cultured group (Fig. 1B). We also compared APAP-CYS adducts formation, a measure of NAPQI production, between NCP-cultured and conventionally-cultured HepG2 cells. The chromatograph
charts of HPLC-ECD analysis are shown in Fig. 1C. A peak of the standard sample of APAP-CYS was observed, and the retention time was approximately 7.3 min (Fig. 1C, lower lane). Although we observed a peak of tyrosine, an internal standard of cellular proteins, APAP-CYS adducts were not detected in the conventionally cultured group at 24 h after APAP exposure. In contrast, a peak of APAP-CYS was identified in the NCP-cultured group at 24 h after APAP treatment, and the amount of APAP-CYS was approximately 0.177 μg/mg cellular protein. Next, we compared the effects of APAP on cell viability in HepG2 cells 48 h after APAP exposure between the NCP-cultured and conventionally cultured groups (Fig. 1D). In the NCP-cultured HepG2 cells, APAP (5 – 20 mM) significantly decreased cell viability 48 h after treatment in a dose-dependent manner. Although a significant decrease was also observed with 20 mM APAP, statistically significant differences were not observed when the cells were treated with 15 mM or less of APAP in the conventionally cultured group. In addition, there were significant differences in cell viability between the conventionally and NCP-cultured groups.

**Time-course parameters of APAP-induced liver injury in NCP-cultured HepG2 cells**

Distinctive changes of APAP-induced liver injury, such as decreases in intracellular GSH and mitochondrial membrane potential and phosphorylation of JNK during cell injury, were evaluated in NCP-cultured HepG2 cells. As shown in Fig. 2A, 15 mM APAP decreased the cell viability of NCP-cultured HepG2 cells. Statistically significant differences were observed at 12 h after APAP exposure. At 48 h after APAP exposure, we also observed a decrease in green fluorescence (calcein-AM, index of viable cells) and increase in red fluorescence (PI, index of dead cells). Subsequently, we observed a decrease in cellular total GSH levels in the 15 mM APAP–treated NCP-cultured HepG2 cells in a time-dependent manner. Statistically significant differences were observed at 6 h after exposure (Fig. 2B). As shown in Fig. 2C, we observed a decrease in red/green fluorescence caused by APAP exposure in the NCP-cultured HepG2 cells in the JC-1 assay. A significant decrease in red/green fluorescence was observed at 12 h after exposure. As shown in Fig. 2D, phospho-JNK was increased by APAP at 6 h after exposure.

**Effects of pharmacological agents against APAP-induced cell injury in NCP-cultured HepG2 cells**

As shown in Fig. 3A, the preventative effects of NAC, a clinical antidote against APAP-induced hepatotoxicity, were not observed in the conventionally cultured HepG2 cells and did not protect against the decrease in cell viability induced by APAP (20 mM). In contrast, NAC significantly prevented a decrease in cell viability induced by APAP (15 mM) in the NCP-cultured HepG2 cells in a dose-dependent manner (Fig. 3B). We also observed an increase in green fluorescence (viable cells) and decrease in red fluorescence (dead cells) in the APAP + 100 μM NAC–treated group compared with the APAP-treated group (Fig. 3C).

We also assessed other pharmacological inhibitors on the mechanism of APAP-induced hepatocyte toxicity that exists in rodents and humans in vivo. Treatment with SP600125 (a JNK inhibitor) and CyA (a MPT inhibitor) attenuated the decrease in APAP-induced cell viability in the NCP-cultured HepG2 cells (Fig. 4).

In addition, we examined the effects of pharmacological inhibitors on the parameters of APAP-induced cell injury in NCP-cultured HepG2 cells. The decrease in GSH content in the cells induced by APAP treatment was significantly suppressed with 100 μM NAC treatment (Fig. 5A). In contrast, treatment with 10 μM SP600125 (a JNK inhibitor) and 100 nM CyA (a MPT inhibitor) did not prevent a decrease in GSH content (Fig. 5A). As shown in Fig. 5B, the decrease in red/green fluorescence of the JC-1 dye, a measure of mitochondrial membrane potential caused by APAP exposure, was significantly suppressed by NAC or CyA treatment. Meanwhile, SP600125 did not show any effects on the decrease in red/green fluorescence of the JC-1 dye. The increase in the expression of phospho-JNK induced by APAP was eliminated by NAC, SP600125, or CyA treatment (Fig. 5C).

**Discussion**

We demonstrated that NCP-cultured HepG2 cells showed higher susceptibility to APAP-induced cell injury compared with conventional monolayer plate cultured cells, as well as higher expression of CYP2E1 mRNA and protein. The significant decrease in GSH contents and mitochondrial membrane potential, and phosphorylation of JNK, which have been reported as critical events for the development of APAP-induced hepatotoxicity, were induced by APAP in NCP-cultured HepG2 cells. In addition, APAP-induced cell injury was significantly attenuated by NAC, a GSH precursor used as an approved antidote for clinical APAP-induced hepatotoxicity, in NCP-cultured HepG2 cells compared with conventionally-cultured HepG2 cells. Furthermore, CyA, an inhibitor of MPT, and SP600125, a JNK inhibitor, prevented APAP-induced cell injury in NCP-cultured HepG2 cells. These results indicate that the APAP-treated NCP-cultured HepG2 cells underwent typical APAP-induced hepatotoxic mechanistic effects similar
Fig. 2. Time-courses of the parameters of APAP-induced toxicity in NCP-cultured HepG2 cells. HepG2 cells cultured in NCP were treated with 15 mM APAP for each time. A) Cells were exposed to 15 mM APAP for 0 – 48 h, and cell viability was measured using the WST-8 assay. The fluorescence micrograph by double staining to calcein-AM (green, index of viable cells) and PI (red, index of dead cells) were taken at 0 or 48 h after APAP exposure. Scale bar = 50 μm. B) Glutathione content in cells exposed to 15 mM APAP for 0 – 48 h. Glutathione content was significantly decreased after 6 – 48 h APAP exposure compared with 0 h. C) Percent of the ratio of red-to-green fluorescence of the JC-1 dye at 0 – 48 h after APAP exposure. The ratio was significantly decreased after 12 – 48 h APAP exposure compared with 0 h. D) p-JNK and JNK protein after 0 – 12 h APAP exposure by western blotting analysis. Each bar represents the mean ± S.D. (n = 4 – 6).*P < 0.05, **P < 0.01 compared with 0 h APAP exposure.

Fig. 3. Effect of NAC against APAP-induced cell injury in conventionally and NCP-cultured HepG2 cells. A) Conventionally cultured HepG2 cells were exposed to 20 mM APAP in the presence or absence of NAC (1 – 100 μM). Cell viability was measured at 48 h after APAP exposure using the WST-8 assay. Each bar represents the mean ± S.D. (n = 6). B) NCP-cultured HepG2 cells were exposed to 15 mM APAP in the presence or absence of NAC (1 – 100 μM). Cell viability was measured at 48 h after APAP exposure using the WST-8 assay. NAC prevented the decrease in cell viability induced by APAP exposure in NCP-cultured HepG2 cells in a dose-dependent manner. Each bar represents the mean ± S.D. (n = 6). **P < 0.01 compared with the APAP-treated group. C) Fluorescence micrographs of double staining to calcein-AM (green, index of viable cells) and PI (red, index of dead cells) were taken after 48 h APAP exposure with or without 100 μM NAC. Scale bar = 100 μm.
A Human Model of Acetaminophen Hepatitis

Fig. 4. Effects of CyA and SP600125 against APAP-induced cell injury in NCP-cultured HepG2 cells. NCP-cultured HepG2 cells were exposed to 15 mM APAP with either the presence or absence of CyA (1 – 100 nM) or SP600125 (0.1 – 10 μM) for 48 h, and cell viability was measured using the WST-8 assay. APAP (black bar) induced a decrease in cell viability, and this reduction was significantly inhibited by 100 nM CyA (left hatched bar) and 10 μM SP600125 (right hatched bar). Each bar represents the mean ± S.D. (n = 6). *P < 0.05, **P < 0.01, compared with the APAP-treated group.

Fig. 5. Effect of NAC, SP600125, and CyA on the parameters of APAP-induced cell injury in NCP-cultured HepG2 cells. A) Glutathione content in cells exposed to 15 mM APAP with or without NAC (100 μM), SP600125 (10 μM), or CyA (100 nM) for 12 h. Although the decrease in glutathione content induced by APAP exposure was significantly attenuated by NAC treatment, SP600125 or CyA did not show any effects. B) The ratio of red-to-green fluorescence of the JC-1 dye at 12 h after APAP with or without NAC, SP600125, or CyA treatment. The decrease in ratio induced by APAP exposure was significantly prevented by NAC or CyA treatment. Each bar represents the mean ± S.D. (n = 4). *P < 0.05, **P < 0.01, compared with the control; #P < 0.05, ##P < 0.01, compared with APAP exposure. C) Determination of p-JNK and JNK protein after 6 h APAP with or without NAC, SP600125, or CyA treatment by western blotting analysis. Treatment of NAC, SP600125, or CyA inhibited the increase in p-JNK expression induced by APAP exposure.

to that observed in humans and rodents.

In general, HepG2 cells have been considered an insufficient in vitro model to study the mechanisms of APAP-induced liver injury (14, 15). Recently, Nakamura et al. (16) demonstrated that the 3D-culture method enhances hepatocyte-specific functions of hepatocytes, including drug-metabolizing enzyme activities in HepG2 cells. They also identified higher sensitivity to acetaminophen cytotoxicity in 3D-cultured HepG2 cells compared with conventional monolayer cultured HepG2 cells and suggested that this 3D-cultured HepG2 cell system may be useful as a human model of APAP-induced hepatotoxicity. However, the pathophysiological relevance of the mechanisms of APAP-induced cytotoxicity observed in 3D-cultured HepG2 cells remains a missing piece of the puzzle (16, 17). Our current results of APAP-induced GSH depletion, mitochondrial dysfunction, and JNK pathway activation as well as cytoprotective action by NAC, CyA, and SP600125 observed in the NCP-HepG2 system, a 3D-cultured method, may explain mechanisms of cytotoxicity more accurately than conventionally cultured methods and be more useful in toxicological and pharmacological safety assessment.

Based on these facts, we advocate that the NCP-HepG2 system, which requires neither specialized techniques nor expensive apparatus, may be a valuable tool as a convenient human in vitro model of APAP-induced hepatotoxicity.

Many studies in this field have used rodent models for defining mechanisms of APAP-induced hepatotoxicity and for screening drug candidates against liver injury (8, 22 – 24). However, considerable differences may
exist in the mechanism of APAP-induced hepatotoxicity between rodents and humans. For example, although increased plasma aminotransferase activity is observed within 2 – 6 h of administration of a toxic dose of APAP [with peak activity achieved around 12 – 24 h in rodents (25)], an increased plasma transaminase activity is rarely observed before 12 – 24 h following ingestion and does not peak until 48 – 72 h in humans (26). In the present study, the first statistically significant decrease in cell viability was observed at 12 h after APAP treatment in NCP-cultured HepG2 cells, and a noticeable decrease was observed at 24 and 48 h after APAP treatment. The time course of cell injury and changes in GSH content and mitochondrial membrane potential induced by APAP in the NCP-cultured HepG2 system is not observed in rodents but was similar to that in humans and HepaRG cells, which is a human hepatoma cell line with high activity of CYP2E1 enzymes and has been reported to be useful as a human model for APAP-induced liver injury (27). In contrast, cell injury, decreased GSH content, and mitochondrial membrane potential induced by APAP treatment were observed within 3 – 5 h in a mouse primary hepatocyte system (28, 29). These facts also support the potential of the NCP-HepG2 system as a human in vitro model of APAP-induced hepatotoxicity.

NAC, a precursor of GSH that causes NAPQI detoxification, has been shown to produce remarkable effects against APAP-induced hepatotoxicity in rodents and humans (22, 30, 31). In the present study, NAC demonstrated such preventive effects against APAP-induced cell injury in NCP-cultured HepG2 cells but did not exhibit any effects on cell injury in the conventionally-cultured HepG2 cells. In addition, we demonstrated that SP600125 and CyA, which showed protective effects against APAP-induced hepatotoxicity in rodents in vivo (29, 32, 33), also attenuated APAP-induced cell injury in NCP-cultured HepG2 cells in vitro. These results indicate that APAP-induced cellular injury in this NCP-cultured HepG2 system share the same cell death mechanisms of APAP-induced hepatotoxicity in humans and rodents. In addition, these facts support the relevancy of this in vitro model of APAP-induced hepatotoxicity and suggest the utility of this APAP-induced hepatotoxicity model as an in vitro screening system of drug candidates (antidotes) against APAP-induced liver injury.

Molecular mechanisms of APAP-induced hepatocyte cell death have been evaluated by many researchers and seem to have become clearer (Supplementary Fig. 2: available in the online version only) (9, 34, 35). The toxic reactive metabolite NAPQI is detoxified by GSH resulting in extensive hepatic GSH depletion. Concurrently, an increasing amount of NAPQI binds to cellular proteins including mitochondrial proteins. Mitochondrial protein adducts cause generation of reactive oxygen species in mitochondria to induce the activation of glycogen synthase kinase-3b (GSK-3b), mixed-lineage kinase-3 (MLK3), and apoptosis signal-regulating kinase-1 (ASK-1) (36). The early phase of JNK activation in APAP-induced hepatotoxicity most likely involves GSK-3b and MLK3, and the rate phase seems to involve ASK-1 through MAP Kinase Kinase (MKK) 4/7 activation (33, 37). JNK phosphorylation amplified further mitochondrial damage and induced subsequent mitochondrial permeability transition (MPT). The feedback loop between JNK pathway activation and mitochondrial damage may be involved in further MPT induction and mitochondrial damage (38). The significant opening of the MPT pore in the mitochondria with collapse of the membrane potential induce the release of mitochondrial intermembrane proteins such as apoptosis inducing factor (AIF) (10). The collapse of the mitochondrial membrane potential with ATP depletion and nuclear degradation are key events leading to hepatocyte necrosis (39). It has been reported that knockout mice of ASK-1, JNK1, JNK2, or AIF gene showed low hepatotoxicity against APAP injection (40). In addition, pharmacological inhibition of JNK or MPT using SP600125 or CyA, respectively, showed marked inhibition of hepatotoxicity induced by APAP in mice (40, 41). Therefore, these molecules and events may play an important part in the development of cellular injury in APAP-induced hepatotoxicity. In this study, we demonstrated that SP600125 or CyA showed cytoprotective effects against APAP-induced cell injury in NCP-cultured HepG2 cells and suggested that activation of the JNK pathway and MPT, at least in part, play important roles in the development of cell injury in our model.

In this study, a GSH precursor, NAC, prevented GSH depression, decrease in mitochondrial membrane potential, and JNK phosphorylation as well as cell injury induced by APAP in NCP-cultured HepG2 cells. As mentioned above, GSH depression is one of the most upstream events of the mechanisms of APAP-induced hepatotoxicity. NAC facilitated NAPQI detoxification through GSH supplementation and may be able to inhibit changes in all of the parameters examined in this study. Besides, other mechanisms, such as antioxidant effects (42), anti-inflammatory effects (43) may be involved in the attenuating effects of NAC against APAP hepatotoxicity. Although the MPT inhibitor CyA significantly attenuated the decrease in mitochondrial membrane potential and JNK phosphorylation, GSH depression was not prevented by CyA. These results indicated that the preventive effects of CyA against APAP-induced cell injury were of little relevance to
NAPQI production or GSH content. The reason for the inhibiting mechanisms of JNK phosphorylation by CyA may be discontinuation of the feedback loop between JNK pathway activation and mitochondrial damage. In fact, phosphorylation of JNK and subsequent mitochondrial damage is not one-way; mitochondrial damage may induce further JNK phosphorylation (2nd hit), and it may amplify the decrease in mitochondrial membrane potential or mitochondrial damage in APAP-induced hepatotoxicity (34, 44) (Supplementary Fig. 2). By contrast, the JNK inhibitor SP600125 did not prevent the decrease in mitochondrial membrane potential induced by APAP exposure in NCP-cultured HepG2 cells, Nevertheless, JNK phosphorylation was almost completely prevented. Although the exact mechanisms of the lack of response to the mitochondrial membrane potential by SP600125 are unclear, less selectivity to the JNK1 or JNK2 of SP600125 may be involved. Bourdi et al. (45) demonstrated that JNK2 knockout mice had a higher mortality rate compared with wild-type mice and suggested that JNK2 is involved in the protection against APAP-induced hepatotoxicity through the stimulation of hepatocyte proliferation and repair. Recently, Ni et al. (46) advocated that the effects of JNK inhibitors should be carefully evaluated on both injury mechanisms and regeneration. Therefore, further study will be warranted using selective JNK1 or JNK2 inhibition to solve this issue.

In the present study, SP600125 and CyA were dissolved in DMSO and applied in the culture medium. DMSO has been commonly used in biological studies as an inert solvent to dissolve drugs with low water solubility. However, DMSO has been known to attenuate liver injury induced by APAP overdose in rodents, and often the facts confound evaluation of the protective effects of compounds with poor water solubility against APAP-induced liver injury (47, 48). In this study, we also confirmed that the treatment with 5% DMSO attenuated the increase in serum alanine aminotransferase levels and hepatocellular necrosis induced by APAP (400 mg/kg, i.p.) in mice (Supplementary Fig. 3: available in the online version only). In contrast, 0.01% – 0.1% DMSO did not show any effects on APAP-induced cellular injury in NCP-cultured HepG2 cells (Supplementary Fig. 4: available in the online version only). One postulated reason for this is a slower NAPQI generation rate in NCP-cultured HepG2 cells in vitro than that in rodents in vivo, quantitated as the GSH depletion rate caused by CYP inhibition by DMSO. Alternatively, Masson et al. (49) reported that DMSO can reduce APAP-induced hepatotoxicity through the activation of natural killer T cells and natural killer cells in mice. These conflicting results of the effects of DMSO between mice and the NCP–HepG2 system may be due to the existence of a type of cells other than hepatocytes, such as natural killer cells. Therefore, it should be noted that if the test compound protects against APAP-induced liver injury only through the modulation of inflammatory cell activity, such as the inhibition of Kupffer cells or natural killer cells, it would not be able to exert a protective action in this in vitro model. These results suggest that the APAP-induced hepatotoxicity model using the NCP-cultured HepG2 system can evaluate the direct effects of test compounds on hepatocytes and that DMSO can be used as a solvent for lipophilic compounds in this system.

Other reports suggest that APAP-induced hepatotoxicity in humans has demonstrated mechanism-related consistency between the in vitro model compared with in vivo models or in patients (27, 50, 51). However, no reports have examined whether antidotes of APAP-induced hepatotoxicity, such as NAC, SP600125, and CyA, could attenuate APAP-induced cellular injury in vitro models. In addition, we also examined the effects of antidotes on APAP-induced cell injury in an NCP-cultured HepG2 cell system, which suggests its usefulness as a screening system for drug candidates against APAP-induced hepatotoxicity. Furthermore, our system using HepG2 cells can be conveniently and easily used compared with other expensive cells such as Hepa RG cells or human primary hepatocytes. These points are characteristic of our in vitro human APAP-induced hepatotoxicity model compared with other in vitro human models. Of course, further study will be needed to demonstrate the usefulness of the model using NCP-cultured HepG2 cells.

In summary, we have demonstrated that NCP-cultured HepG2 cells show key mechanistic features of APAP-induced hepatotoxicity, such as a decrease in intracellular GSH and mitochondrial membrane potential, activation of JNK, and cellular injury, after APAP treatment. In addition, we showed that antidotes of APAP-induced hepatotoxicity, such as NAC, SP600125, and CyA, could attenuate APAP-induced cellular injury in NCP-cultured HepG2 cells. These results suggest that cellular injury induced by APAP treatment using the NCP-cultured HepG2 system is a useful human model to study toxicity mechanisms and to screen drug candidates against APAP-induced hepatotoxicity.

Acknowledgments

The authors gratefully acknowledge the financial support from the Japan Society for the Promotion of Science (Grants-in-Aid for Scientific Research for Young Scientists (B), No. 21790524 and 23790603 to Y.I.).
Conflicts of Interest

The authors declare that they have no competing interests.

References

31. Blackford MG, Felter T, Gothard MD, Reed MD. Assessment of the clinical use of intravenous and oral N-acetylcysteine in...


