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Fluorometric assessment of acetaminophen-induced toxicity in rat hepatocyte spheroids seeded on micro-space cell culture plates

Seigo Sanoh [a,*], Masataka Santoh [a], Masashi Takagi [b], Tatsuya Kanayama [a], Kazumi Sugihara [c], Yaichiro Kotake [a], Yoko Ejiri [d], Toru Horie [e], Shigeyuki Kitamura [f], Shigeru Ohta [a]

[a] Graduate School of Biomedical and Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan
[b] Faculty of Pharmaceutical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan
[c] Faculty of Pharmaceutical Sciences, Hiroshima International University, 5-1-1 Hirokoshinkai, Kure, Hiroshima 737-0112, Japan
[d] Tsukuba Research Center, Kuraray Co., Ltd., 41 Miyukigaoka, Tsukuba, Ibaraki 305-0841, Japan
[e] De Three Research Laboratories, 25-4 Saigo, Tsukuba, Ibaraki 305-0036, Japan
[f] Nihon Pharmaceutical University, 10281 Komuro, Ina-machi, Kitaadachi-gun, Saitama 362-0806, Japan

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ABSTRACT

Hepatotoxicity induced by the metabolic activation of drugs is a major concern in drug discovery and development. Three-dimensional (3-D) cultures of hepatocyte spheroids may be superior to monolayer cultures for evaluating drug metabolism and toxicity because hepatocytes in spheroids maintain the expression of various metabolizing enzymes and transporters, such as cytochrome P450 (CYP). In this study, we examined the hepatotoxicity due to metabolic activation of acetaminophen (APAP) using fluorescent indicators of cell viability and intracellular levels of glutathione (GSH) in rat hepatocyte spheroids grown on micro-space cell culture plates. The mRNA expression levels of some drug-metabolizing enzymes were maintained during culture. Additionally, this culture system was compatible with microfluorometric imaging under confocal laser scanning microscopy. APAP induced a decrease in intracellular ATP at 10 mM, which was blocked by the CYP inhibitor 1-aminobenzotriazole (ABT). APAP (10 mM, 24 h) decreased the levels of both intracellular ATP and GSH, and GSH-conjugated APAP (APAP-GSH) were formed. All three effects were blocked by ABT, confirming a contribution of APAP metabolic activation by CYP to spheroid toxicity. Fluorometric imaging of hepatocyte spheroids on micro-space cell culture plates may allow the screening of drug-induced hepatotoxicity during pharmaceutical development.

1. Introduction

Evaluation of hepatotoxicity is a critical step in new drug development because reactive drug metabolites produced by liver cytochrome P450 (CYP) can bind and inactivate numerous cell macromolecules, leading to adverse and often idiosyncratic drug reactions. Various approaches using in vivo animal models and in vitro cell cultures have been developed to test for possible drug-induced hepatotoxicity in humans, including liver slices, isolated hepatocytes, primary cultured hepatocytes, liver-derived cell lines, and subcellular fractions such as liver S-9, microsomes, and mitochondria. Each of these models has several drawbacks that hamper the extrapolation of results to the human liver in vivo. Although easily harvested from experimental animals, isolated hepatocytes in suspension can be used only for short-term drug exposure. Primary cultured hepatocytes can be used for longer drug exposures, additionally, they form cell–cell contacts as observed in vivo. Furthermore, monolayer culture improves long-term survival compared with suspended hepatocytes. However, activities of drug-metabolizing enzymes are often reduced during monolayer cultivation (Gómez-Lechón et al., 2010; Meng, 2010).

A micro-patterned human hepatocyte co-culture system was recently developed for the screening of drug hepatotoxicity that maintains the expression of drug-metabolizing enzymes during long-term culture (Khetani and Bhatia, 2008), and thus, this system may provide a better model to predict human drug responses (Ukairo et al., 2013; Wang et al., 2010). Three-dimensional (3-D) culture models, such as the gel-entrapment hepatocyte culture,

Abbreviations: CYP, cytochrome P450; 3-D, three-dimensional; APAP, acetaminophen; NAPQI, N-acetyl-p-benzoquinone imine; GSH, glutathione; ROS, reactive oxygen species; UGT, UDP-glucuronosyltransferase; SULT, sulfotransferase; ABT, 1-aminobenzotriazole; mBCl, monochlorobimane.

* Corresponding author. Tel.: +81 82 257 5327; fax: +81 82 257 5329.
E-mail address: sanoh@hiroshima-u.ac.jp (S. Sanoh).

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3-D perfused bioreactors, and 3-D hepatic co-cultures of primary parenchymal and non-parenchymal hepatocytes, have also been developed to more accurately predict drug metabolism and hepatotoxicity in humans (Darnell et al., 2011; Dash et al., 2009; Fey and Wrzesinski, 2012; Kostadinova et al., 2013; Meng, 2010; Yin et al., 2013). These 3-D culture models are known to produce a cellular microenvironment that partially mimics that of liver tissue in vivo, including the development of a more elaborate extracellular matrix and pathways for intercellular communication.

A micro-space cell culture plate was recently developed by Kuraray Co., Ltd. (Tokyo, Japan) as a convenient tool for the formation and cultivation of cell spheroids. Uniformly-sized spheroids can be formed after seeding because these plates have regularly spaced square compartments of equal volume (200 μm-length × 200 μm-width × 50 μm-depth) etched into the bottom surface and coated with poly-L-lysine (Fig. 1). The expression levels of several CYP enzymes were higher in human hepatocyte spheroids grown on these micro-space cell culture plates compared with those in human hepatocytes grown on 2-D monolayer cultures on collagen-coated flat plates (Nakamura et al., 2011; Nishimura et al., 2010). Furthermore, these spheroid cultures exhibited a stable expression of membrane transporters and drug-metabolizing enzymes such as transferases (Nishimura et al., 2011).

Acetaminophen (APAP) is a widely used analgesic and antipyretic drug; however, APAP may cause hepatotoxicity at higher doses. At therapeutic doses (⩾200 mg), the APAP metabolite N-acetyl-p-benzoquinone imine (NAPQI) is efficiently detoxified by conjugation to glutathione (GSH) (Fig. 2), but the high levels of NAPQI generated by APAP over-dose bind to cellular proteins and deplete GSH, leading to accumulation of reactive oxygen species (ROS) and ensuing oxidative cell death. Furthermore, mitochondrial dysfunction caused by APAP results in depletion of cellular ATP (Damsten et al., 2007; James et al., 2003; McGill and Jaeschke, 2013; Sakatis et al., 2012).

Nakamura et al. (2011) found that the HepG2 or primary human hepatocyte spheroid viability on micro-space cell culture plates was lower than that of monolayer cultures on collagen-coated plates during APAP treatment, possibly the result of an enhanced expression of the NAPQI-generating enzyme CYP2E1. Similarly, Schyschka et al. (2013) reported higher APAP toxicity in a 3-D model compared with that in hepatocyte monolayers, again likely due to the induced expression of CYP2E1 as well as reduced superoxide dismutase (SOD) activity. Finally, Shen et al. (2006) reported higher APAP toxicity and CYP2E1 activity in gel entrapped hepatocytes.

Mitochondria damage, oxidative stress, and intracellular changes in GSH associated with drug-induced liver injury can be assessed using various fluorescent probes (Xu et al., 2008). McGill et al. (2011) measured APAP toxicity of HepaRG cells using fluorescence probes sensitive to ROS generation and cell viability, whereas Lan et al. (2010) measured cell viability using live/dead fluorescence probes in 3-D cultures of HepG2 cells encapsulated in alginated hydrogels.

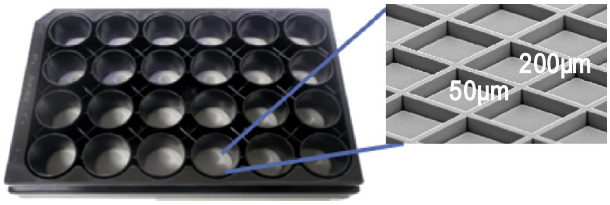

Fig. 1. The micro-space cell culture plate (Elplasia®; Kuraray Co., Ltd.) used in this study. Each well has regularly spaced square compartments (200 μm-length × 200 μm-width × 50 μm-depth).

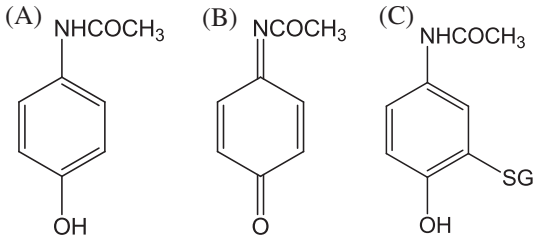

Fig. 2. Chemical structures of (A) acetaminophen (APAP) and its metabolites, (B) N-acetyl-p-benzoquinone imine (NAPQI) and (C) APAP-glutathione (APAP-GSH).

In the present study, we confirmed the expression of CYPs and conjugation enzymes in spheroids derived from primary rat hepatocytes seeded onto micro-space cell culture plates. We then examined changes in the intracellular levels of ATP, GSH, and APAP-GSH following APAP exposure. We conclude that microfluorometry of hepatocyte spheroids using micro-space cell culture plates is a convenient approach for measuring cell viability and intracellular responses during the application of hepatotoxic drugs.

2. Methods

2.1. Materials

APAP, and 1-aminobenzotriazole (ABT) were purchased from Sigma–Aldrich (St. Louis, MO, US). APAP-glutathione (APAP-GSH) standard was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). The calcein-AM vital cell imaging probe was purchased from Dojindo Laboratories, Kumamoto, Japan. A cell-based assay kit containing the thiol-sensitive fluorophore monochlorobimane (mBCl) for GSH measurements was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). All other chemicals and reagents used in this study were of analytical grade.

2.2. Animals

Crj:CD (SD) rats (7 weeks old) were obtained from Charles River Laboratories, Japan. Hepatocytes were isolated using collagenase perfusion (Berry and Friend, 1969). The viability of the suspended hepatocytes used to seed culture plates for spheroid growth was of >90%, as determined by trypan blue exclusion. The present study was approved by the animal ethics committee of Hiroshima University.

2.3. Cell culture

Primary rat hepatocytes were cultured in Dulbecco's modified Eagle's Nutrient Mixture F12 Ham medium in a humidified atmosphere (5% CO_2 + 95% O_2 at 37 °C). The medium contained 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 10 mM nicotinamide, 50 μM β-mercaptoethanol, 1.5 μM dexamethasone, 520 μM L-ascorbic acid, 1 μg/mL insulin, and 5 mM HEPES. Hepatocytes were seeded in 500-μL medium at 2.5×10^5 cells/well in 24-well micro-space cell culture plates coated with 0.01% poly-l-lysine. The medium was changed once a day.

2.4. mRNA expression of drug metabolizing enzymes

Total RNA was extracted from suspended hepatocytes at day 0, isolated from rats, and cultivated hepatocytes at days 1, 3, 5, 7, and 9 after plating using the SV Total RNA Isolation System (Promega, Fitchburg, WI) according to the manufacture's procedure.