Master of Science

Usefulness of 3D liver organoid derived from human liver to predict drug-induced phospholipidosis

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of the University of Ulsan

Department of Medicine
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Usefulness of 3D liver organoid derived from human liver to predict drug-induced phospholipidosis

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Usefulness of 3D liver organoid derived from human liver to predict drug-induced phospholipidosis

This certifies that the master's thesis

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Abstract

Drug-induced phospholipidosis (PL) is a phospholipid storage disorder caused by the formation of phospholipid-drug complexes in lysosomes. PL caused by the drug in human can be a problem in the pharmaceutical development. It is not certain that PL in an animal is toxicologically relevant to human due to the metabolic differences between species. To screen out compound with potential inducing PL in human, cell-based *in vitro* assays using various types of cells from human origin have been reported. We established three-dimensional (3D) human liver organoid according to the method as described previously (Broutier et al., 2016). Drug-induced PL has been testified in 3D human liver organoid and HepG2 cells cultured by the conventional method by systemically examining cellular alterations including viability change, albumin content, microscopic examination with special immunostaining and transmission electron microscope and gene expression change specialized to PL. As a result of various evaluation about characteristics of the organoids and HepG2, organoids that underwent differentiation to hepatocytes showed more similar nature of hepatocyte than HepG2 cells. 3D human liver organoids survived in high dose PL-inducing drug rather than HepG2 cells for 48 h of drug incubation. 3D liver organoids kept the capacity of albumin secretion more stable than monolayered HepG2 cells. In the morphological evaluation of PL, the more potent PL-inducing drug administrated, the more cytoplasmic vacuoles were observed in organoids and HepG2 cells, and to a greater extent in organoids. In immunohistochemistry of lysosome-associated membrane protein 2 (LAMP-2), a special marker for lysosome membranes, the expression of LAMP-2 in organoids seemed more apparent than that in HepG2 cells in the same condition. PL-characteristic lamellar bodies were observed in only in the amiodarone-treated organoids under the transmission electron microscopic examination. Our present study showed that 3D cultured human liver organoids were more sensitive to drug-induced PL and less affected by cell death toxicity than HepG2 grown in a monolayer. Since PL is chronic cellular alteration rather than cytotoxic acute toxicity, these results support well that 3D cultured organoids reflected the metabolite-mediated hepatotoxicity *in vivo*. Conclusively, 3D human liver organoids appeared to be a relevant system for evaluating phospholipidogenic effects of compounds. As cell-based

approaches, 3D human liver organoids can be used early in the drug development process to identify chemicals with the potential to induce PL.

Keywords: Drug-induced phospholipidosis, 3D human liver organoid, HepG2

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1 Introduction

Drug-induced phospholipidosis (PL) is a phospholipid storage disorder caused by the formation of phospholipid-drug complexes in lysosomes, which is characterized as lamellar bodies under the transmission electron microscope (Asaoka et al., 2013). After the first case of drug-induced PL in human was reported, more than fifty marketed drugs have been described that they have potential to induce PL in human. Most of drugs involved this process categorized to cationic amphiphilic drugs (CADs) which have side chains with a charged hydrophilic ring and hydrophobic regions known to induce PL (Halliwell, 1997; Mark J. Reasor & Kacew, 2001). Although the molecular mechanism underlying CAD-induced PL is still not fully understood, it is considered to be associated with the formation of an indigestible phospholipid-drug complex, the suppression of phospholipase activity, and an imbalance between production and degradation of phospholipid (Halliwell, 1997; Mark J. Reasor & Kacew, 2001).

In the course of pharmaceutical development, PL is detected quite frequently as histological findings during exploratory preclinical *in vivo* studies as features like cytoplasmic vacuolation or cells with foamy appearance. It is generally considered that the accumulation of drugs in cellular organelle, typically lysosomes is an adaptive response rather than adverse effect at the early stage. However, concerning the assessment of adversity in drug administrated chronically, drug-induced PL may impact inferiorly on the success of the project. On the point of regulatory view, drug-induced PL has several toxicological implications. For example, some congenital diseases related to PL exist and depending on the types of mutation of base pair, the disease like Niemann-Pick disease can be fatal. When drug-induced PL appear in lysosomes with concentration over 10 mM (Hostetler, Reasor, & Yazaki, 1985; Nioi, Perry, Wang, Gu, & Snyder, 2007), it may result disruption of cellular membrane integrity and blocking of phospholipid degradation (Halliwell, 1997). If this condition continued by chronic administration, it may impact cellular function or cause cellular death (Mark J Reasor, Hastings, & Ulrich, 2006). Moreover, many other side effects associated with drug-induced PL like QT prolongation, myopathy, hepatotoxicity, nephrotoxicity and pulmonary dysfunction were reported (Tengstrand, Miwa, Hsieh, & toxicology, 2010).

Though drug-induced PL can be found in many preclinical *in vivo* studies, this sort of studies are quite expensive, time-consuming, raise ethical issues and even have less toxicological relevance to humanity. Differences of phospholipase between human and experimental species may be a plausible reason to explain the poor predictive capacity of preclinical animal studies (Nonoyama & Fukuda, 2008). Therefore, it is recommended to identify and screen out those compounds with the potential to lead PL in human in the early discovery stage (Chatman, Morton, Johnson, & Anway, 2009). Since phospholipid is an essential component of the cell membrane, drug-induced PL can be observed in all organs in the body, but lung and liver are most often involved to PL in preclinical *in vivo* studies. To select chemicals which provoke PL in human, cell-based in vitro assays were investigated using various types of cells from human liver origins such as HepG2 cells, HuH7 cells, and human primary hepatocytes. (Bhandari, Figueroa, Lawrence, & Gerhold, 2008; Shahane et al., 2014; van de Water, Havinga, Ravesloot, Horbach, & Schoonen, 2011). About the cells used in cell-based phospholipidosis *in vitro* assays, HepG2 and HuH7 cells are immortal cell lines derived from human hepatocellular carcinoma. Though these cells are cultured easily and the times for doubling are quite short, it is insufficient to say that they represent normal human liver because the cell lines still have characteristics of cancer. By comparison with cell lines, primary human hepatocytes present nature of human normal liver or patient's individuality. It is ideal to use for toxicity test but the viability of primary cells drop quickly so they live in culture medium for about a week at the most. Furthermore, the amount of cells obtained from primary tissue is limited because hepatocytes are fully differentiated and rarely proliferate in culture.

Organoid is recently developed three-dimensional (3D) cell culture system for adult organs which is capable *in vitro* expansion of primary tissues from animal or human (Broutier et al., 2016). Duct cells in adult liver tissue have bipotent stemness to differentiate biliary epithelial cell or hepatocyte. Duct cells form a sphere-shape 3D structure called organoid in *in vitro* extracellular matrices such as Matrigel or Basement Membrane Extract (BME). Those organoids can expand and proliferate by undergoing passage, unlike other primary cells. Once the organoid line is established, it can be maintained more than 6 months until passage over 20. When the organoids reach to the proper number, culture media will be changed to the differentiation media containing hepatocyte growth factors for 10 days. Then, the

organoids will obtain hepatic characteristics by differentiation.Human liver organoids show properties of normal liver or diseased condition. And its unique 3D structure reflects reactions of *in vivo* system rather than conventional two-dimensional (2D) culture method. Organoids are the promising candidate which can be used for toxicity evaluation though no application has been reported yet.

We established 3D human liver organoids according to the method as described previously (Broutier et al., 2016). We used 3D human liver organoids as a tool for predicting drug-induced phospholipidosis. Drug-induced PL was testified in 3D human liver organoids and compared with HepG2 cells that already widely used in *in vitro* screening for PL. Systemical examination for cellular alterations including viability change, albumin secretion, morphological change, protein expression by special immunostaining, ultra-microstructure by transmission electron microscope examination and gene expression change was proceeded to specialize PL. In this study, the feasibility of 3D cultured organoid to predict drug-induced PL was addressed by comparison with monolayered HepG2 cell line.

2 Materials and Methods

2.1 Chemicals

Compounds tested in the assays; amikacin, amiodarone hydrochloride, sertraline hydrochloride, and acetaminophen were purchased from Sigma-Aldrich (St. Louis, MO, USA). Information about each of the PL-inducing drugs can be found in the references (Nioi et al., 2007; Mark J Reasor et al., 2006). We chose amikacin, amiodarone, and sertraline as a weak, moderate and strong PL-inducing drug respectively. Acetaminophen was used as a negative control drug for PL. All other used compounds and reagents were obtained from common commercial sources in analytical grade.

2.2 Cell culture

Human liver organoid

Human liver tissue (0.5-1 cm^3) were obtained from a patient during hepatectomy performed by a trained surgeon at Asan Medical Center, Seoul. The primary human liver tissue was obtained with informed consent and approval by the Institutional Review Board of Asan Medical (approval no. S2017-0969-0003). After surgical excision, the tissue was kept cold at 4 in Hanks' Balanced Salt Solution (HBSS) until processed. Establishment of human liver organoid was conducted following the protocols of Broutier et al. (2016). To isolate duct cells which have bipotent stemness, the tissue was chopped with sterile scissors and digested by collagenase D (Roche) and dispase II (Life Technologies) in sterile Earle's Balanced Salt Solution (EBSS) medium (Thermo Fisher Scientific HyClone), as described previously (Broutier et al., 2016). The isolated duct cells were mixed with Matrigel (BD Biosciences) or reduced growth factor BME 2 (Basement Membrane Extract, Type 2, Pathclear) (Amsbio), and 5,000–10,000 cells were seeded per well in a 24-well plate. After Matrigel or BME had been solidified, 500 μl of culture medium per well was added. Culture media to expand organoids was based on Advanced DMEM/F12 (Invitrogen) and various supplements; 1% N2 and 1% B27 (both from GIBCO), 1.25 mM N-acetylcysteine amide (Sigma), 10 nM gastrin (Sigma), and the growth factors: 50 ng/ml recombinant human EGF (Peprotech), 500 ng/ml R Spondin (Peprotech), 100 ng/ml recombinant human FGF10 (Peprotech), 25 ng/ml

recombinant human HGF (Peprotech), 10 mM nicotinamide (Sigma), 5 uM A83-01 (TGFβ inhibitor) (Tocris Bioscience), and 10 uM forskolin (Tocris Bioscience) were included. To establish the organoid culture, during the first 3 days after isolating duct cells, the medium was supplemented with 25 ng/ml recombinant human Noggin (Peprotech), 100 ng/ml Wnt3a (Peprotech), and 10 uM Y27632 (Sigma Aldrich). Then, the medium was changed into an expansion medium (EM) without Noggin, Wnt3a, Y27632. After single duct cells budded to organoids, passaging was performed in a 1:2-1:4 split ratio once every 5-7 days. For normalization of the number of cells in each well, the organoids were dissociated into single cells using TrypleLE (Gibco) and seeded with the same number per well. The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. For differentiating organoids to hepatocytes, the seeded human liver organoids were kept 5 days under the liver expansion medium supplemented with BMP7 (25 ng/ml) following to change to the differentiation medium (DM); based on advanced DMEM/F12 medium supplemented with 1% N2 and 1% B27 and containing 50 ng/ml recombinant human EGF, 10 nM gastrin (Sigma), 25 ng/ml recombinant human HGF, 100 ng/ml recombinant human FGF19 (R&D), 500 nM A83-01, (10 uM DAPT (Sigma), 25 ng/ml recombinant human BMP7 (Peprotech) and 30 uM dexamethasone (Sigma). Differentiation medium was changed every 2–3 days for a period of 10–12 days.

HepG2 cells

Human hepatocellular carcinoma cell line (HepG2) were kindly obtained from Dr. Shim's laboratory in Asan Institute for life sciences. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 g/ml streptomycin in 175 cm^3 cell culture flasks. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 °C and subcultured every 3 or 4 days using 0.05% trypsin/EDTA in PBS.

2.3 Characterization of human liver organoids

Hepatic marker expression of human liver organoids

Cytochrome P450 proteins are monooxygenases which catalysis many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipid components mainly found in the liver. Cytochrome P450 3A4 (CYP3A4) plays the biggest part in drug metabolism (Zanger & Schwab, 2013). Albumin is one of most common serum protein that secreted from the liver. As a hepatic functional marker of cells, mRNA expression of CYP3A4 and albumin in organoids and HepG2 cells were compared by quantitative real-time Polymerase Chain Reaction (qPCR).

Cells were harvested by Trizol and stored at -80 until their RNA was extracted by the manual protocol using chloroform as described previously (Farrell, 2010). The concentration and purity of the total RNA were determined by measuring absorbance at 260 and 280 nm with a NanoDrop (Thermo Fischer Scientific). To synthesize cDNA, reverse transcription (RT) was performed using RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific). 1000 ng of total RNA was reacted in 20 μl of the mixture including oligo-dT oligonucleotide primer, RevertAid Reverse Transcriptase, and RiboLock RNase inhibitor. Primers used for qPCR are listed in Table 1.

Table 1. Hepatocyte markers and sequences of forward primer, reverse primer for qPCR

Gene symbol	Gene full name	Forward primer	Reverse primer
CYP3A4	cytochrome P450 family 3 subfamily A member 4	CTTCATCCAATGGACTGCATAAAT	TCCCAAGTATAACACTCTACACAGACAA
ALB	albumin	ATGCCCCGGAACTCCTTTTC	CAACAGGCAGGCAGCTTTAT

q PCR was performed by ABI PRISM 7900HT Sequence Detection System (Thermo Fischer Scientific) with the following schedule: 2 min at 50 and 2 min at 95 , followed by 40 cycles of denaturation for 15s at 95 , and annealing and elongation for 1 min at 60 in a

final volume of 10 μL. Relative gene expression levels of two culture systems were normalized to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and calculated by the comparative Ct method.

Glycogen storage

HepG2 cells and organoids were fixed with neutral buffered formalin for 30 min. After washing cells with phosphate-buffered saline (PBS) twice, cells were collected into 1.5 mL tube and spun down gently to protect the cellular structure. Cell pellets were embedded with Histogel, specimen processing gel. Solidified gel block containing cells was transferred to tissue cassettes and standard method to make tissue paraffin-embedded blocks proceeded. To determine glycogen storage in organoids, 3 μm-sectioned slides were stained by Periodic Acid Schiff (PAS) stain. In PAS stain, glycogen, neutral mucins, some epithelial mucins, basement membranes or fungal walls show positive (magenta color).

Hepatic protein expression

The protein encoded by *HNF4A* gene is a nuclear transcription factor that binds DNA and regulates several hepatic genes. This gene may be involved in the development of the liver, kidney, and intestines. To identify whether the protein related to the liver is expressed in organoid or HepG2 cell, immunofluorescence for HNF4α proceeded. 3 μm-sectioned paraffin-embedded cell slides were deparaffinized and antigen retrieval was performed using citrate buffer in 95 ℃ for 15 min. The primary anti-HNF4α antibody (Abcam, ab41898) was incubated 2 hours with 1 μg/mL concentration at room temperature. Alexa Fluor™ 488 goat anti-mouse IgG (Invitrogen, A32723) was incubated in 1:1000 dilution factor for 1 hr in room temperature. DAPI was used for nuclear counterstaining. Images were acquired by the fluorescent microscope (Observer.Z1, ZEISS).

2.4 Exposure of cells to chemicals

The 2D and 3D cultured cells were incubated in the medium including each chemical for 48 hours. When subculturing, dissociated human liver organoid cells were seeded by 10,000 cells per well in 24 well plate for expansion, then differentiation started for 10 days when the

diameter of the organoid does not exceed 200 μm to avoid apoptosis. On the day 10 of differentiation, compounds (5 or 10 μM for amikacin, amiodarone, and sertraline; 25 or 50 μM for acetaminophen) was added to differentiation media. HepG2 cells were seeded on tissue culture treated plates by the suitable counts for each assay. After the cells adhered on the plates incubating overnight, culture media was changed to media with each chemical.

2.5 Cell viability

Organoids and HepG2 cells were plated 10,000 cells per well when PL-inducing drugs were treated. Organoids were incubated with 10 and 20 μM of amikacin, amiodarone, and sertraline. HepG2 cells were incubated with 5 and 10 μM of amikacin, amiodarone, and sertraline. Acetaminophen was treated to both kind of cell culture systems by 25 and 50 μM. Adenosine triphosphate (ATP) content in cells was determined by ATPlite luminescence ATP detection assay system (Perkin Elmer, MA, USA). Cells were treated in 100 μl of cell lysis solution for 5 min following to add substrate reagent for 5 min at room temperature. After adaptation in dark for 10 min, measurement was taken with a luminometer (VICTOR X2, Perkin Elmer, MA, USA). Data were presented as a relative value in percentage to the control culture without drug exposure.

2.6 Determination of albumin content

To assess hepatocyte-specific function, cultured media of HepG2 cells and human liver organoids were collected before and after 48 hours the chemicals treated. The albumin content secreted into the culture medium by monolayered HepG2 cells and 3D human liver organoid was determined by human albumin ELISA kit (Abcam, UK) according to the manufacturer's instructions. Data were expressed as a relative value in percentage to the control culture without drug exposure.

2.7 Evaluation of phospholipidosis

Morphological changes caused by PL-inducing chemicals

To investigate the morphological changes caused by PL-inducing drug treatment, 3 μm-sectioned slides were prepared to perform hematoxylin and eosin stain. Morphological features of HepG2 cell and organoid were evaluated.

Immunohistochemistry for LAMP-2

Lysosome-associated membrane protein 2 (LAMP-2) is a glycoprotein located in the outer membrane of lysosome (Cha, Holland, & August, 1990). The function of LAMP-2 is associated with cell-cell or cell-extracellular matrix adhesion and maturation of autophagic vacuoles (Carlsson, Roth, Piller, & Fukuda, 1988; Tanaka et al., 2000). It is known that LAMP-2 is involved in the direct uptake of cytosolic proteins in lysosome (Carlsson et al., 1988). Immunostaining for LAMP-2 was applied to differentiate PL from other changes such as lipidosis in liver (Ma, Snook, Garrovillo, Johnson, & La, 2017). Paraffin-embedded cell block sections (3 μm) were stained automatically by Discovery XT Autostainer (Ventana Medical Systems). All reagents used for automatic immunohistochemistry were from Ventana Medical Systems. As a primary antibody, anti-LAMP-2 antibody (Invitrogen, PA1-655) was used with 1:200 dilution factor for 40 min. Target antigen was visualized by DAB reaction. Images were acquired by light microscope (BX53, Olympus).

Transmission electron microscopic examination

The organoids were treated 10 μM amiodarone for 48 hours during differnetiation day 10-12 and differentiated organoids with no treatment were prepared for control. The cells were fixed in 2.5% glutaraldehyde solution and stored at 4 for embedding and ultrathin sectioning. The fixed samples were treated with a 1:1 (v/v) mixture of Quetol and dry ethanol for 30 min, and then followed by two treatments in Quetol for 30 and 120 min, respectively. The samples were mounted in the tip of Beem capsules, which were dried overnight in an oven at 74 ◦C. The dried samples were sectioned on a microtome and stained with uranyl acetate for 20 min. Then they were counterstained with palladium for another 4 min, and specimens were viewed under a transmission electron microscope (JEM-1200EX, JEOL, Japan).

Gene expression markers for phospholipidosis

As a toxicogenomic approach to drug-induced PL, fold changes of several gene markers indicating drug-induced PL were measured by qPCR. Sawada and his coworkers reported 17 PL-related gene markers showed significant concordance with lamellar myelin-like body formation using HepG2 cells (Sawada, Takami, & Asahi, 2004). Amiodarone (5, 10 μM) and sertraline (5, 10 μM) were treated to cells for 48 hr. Cells were collected by Trizol and their RNA was extracted. After the concentration and purity of the total RNA measured by NanoDrop (Thermo Fischer Scientific), synthesis of cDNA was performed using RevertAid First Strand cDNA Synthesis kit (Thermo Fisher scientific). 1,000 ng or 5,000 ng of total RNA was reacted in the 20 μl of mixture including oligo-dT oligonucleotide primer, RevertAid Reverse Transcriptase, and RiboLock RNase inhibitor. Primers used for qPCR are listed in Table 2.

Table 2. PL marker genes and sequences of forward primer, reverse primer for qPCR

Gene symbol	Gene full name	Forward primer	Reverse primer
LSS	Lanosterol synthase	GTCCGGTGTCTACTT GAGAAACAG	AGACCCCAGCAATG TTTTCCT
p8	p8 protein (candidate of metastasis 1)	CCTCTATAGCCTGGC CCATTC	CTTCTCTCTTGGTGC GACCTTT
SLC2A3	Solute carrier family 2 (facilitated glucose transporter), member 3	GCTTGAAAAGGTGA CCTTGCA	TGCCTTACTGCCAA CCTACTGTT

q PCR was performed by ABI PRISM 7900HT Sequence Dectection System (Thermo Fischer scientific) with the following schedule: 2 min at 50 and 2 min at 95 , followed by 40 cycles of denaturation for 15s at 95 , and annealing and elongation for 1 min at 60 in a final volume of 10 μL. Relative gene expression levels of two culture systems were normalized to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase

(GAPDH) and calculated by the comparative Ct method. Results are indicated as 2-dCt ($2^{-\Delta\Delta Ct}$) compared with vehicle control.

2.8 Statistical analysis

All values were shown as means±SE. Comparisons between multiple groups were performed with the ANOVA test by SPSS. P-values less than 0.05 were considered statistically significant.

3 Results

3.1 Characterization of human liver organoids

The morphology of HepG2 cell and 3D human liver organoid was compared as shown in Figure 1. Organoids in EM are transparent sphere-shaped structure and expand their size. When organoids went through differentiation, organoids got compact, darkened and each cell have hexagonal-shape. HepG2 cells are monolayered and grow plat.

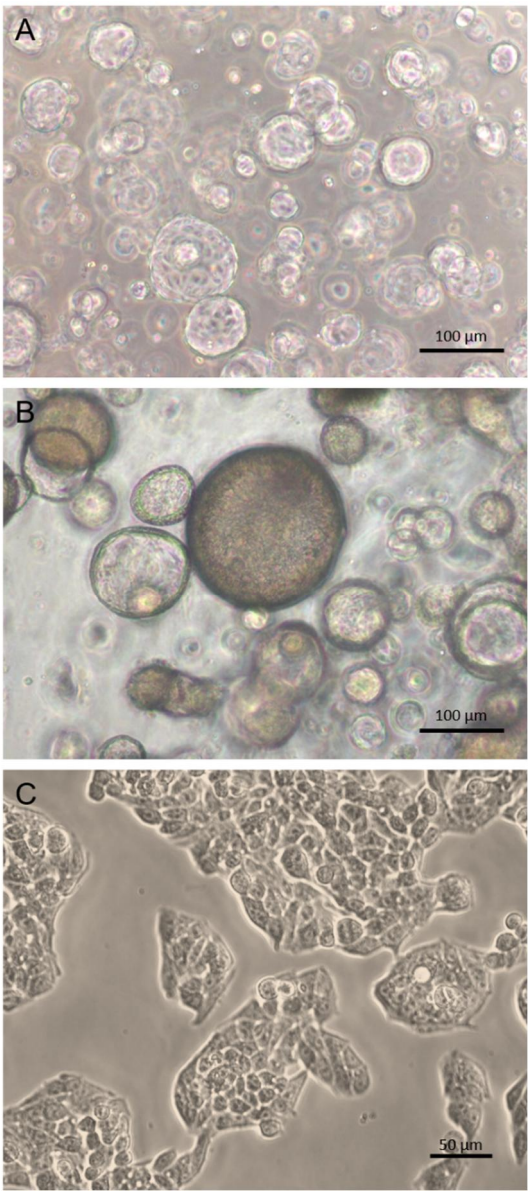

Figure 1. Morphology of cultured hepatocyte system; organoids in EM (A) and DM day 9 (B) and HepG2 cell (C).

Hepatic marker expression of human liver organoids

Gene expressions of CYP3A4 related with metabolic activities were analyzed by qPCR in both hepatocyte culture systems. RNA extract from human liver tissue was used as positive control. The mRNA expression level of CYP3A4 in organoids was little higher than HepG2 cell (Figure 2).

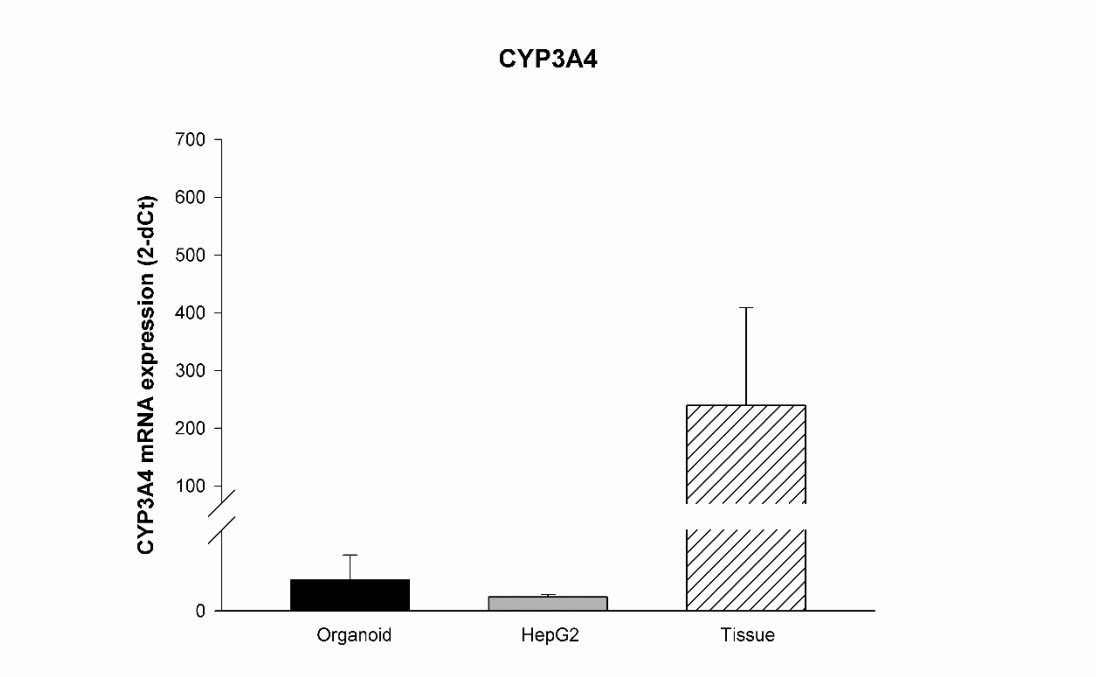

Figure 2. CYP3A4 mRNA expression in organoid and HepG2 determined by qPCR

The mRNA expression level of albumin seemed equivalent between organoids and HepG2 cells (Figure 3). The albumin mRNA expression level in liver tissues was significantly higher than that in both hepatocyte culture systems.

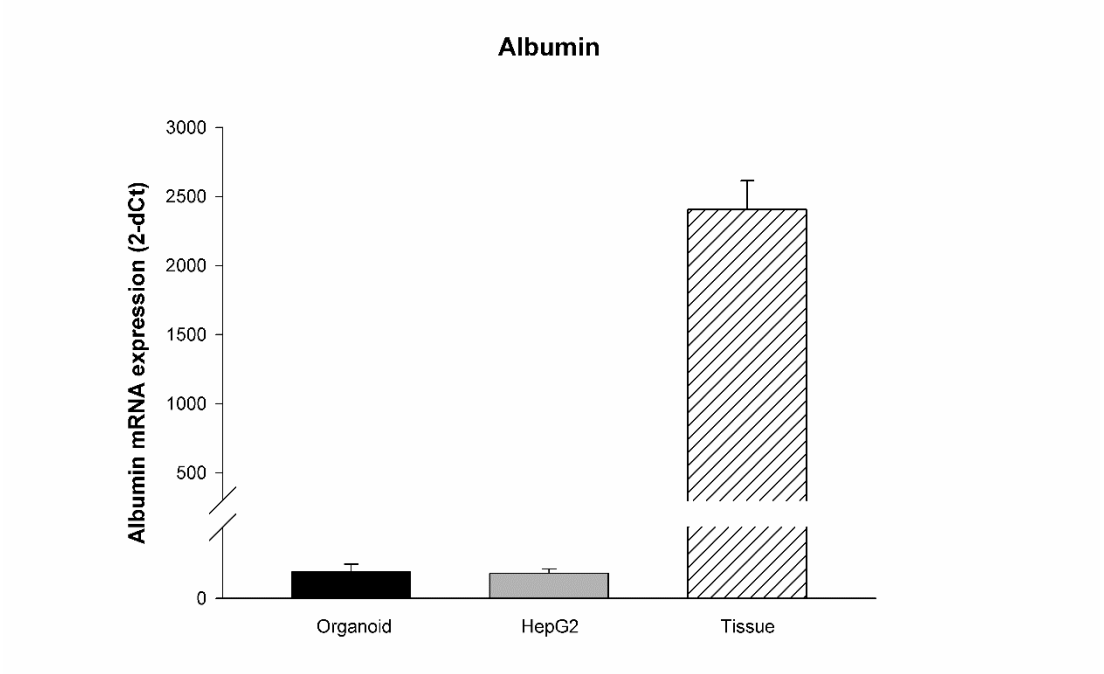

Figure 3. Albumin mRNA expression in organoid and HepG2 determined by qPCR

Glycogen storage

Glycogen accumulation in human liver organoid and HepG2 cell was determined by PAS staining. Organoid showed glycogen accumulation definitely as positive magenta color. The cells consisting organoids contained polysaccharides such as glycogen or mucosubstances such as glycoproteins, glycolipids in cytoplasms as shown in Figure 4.

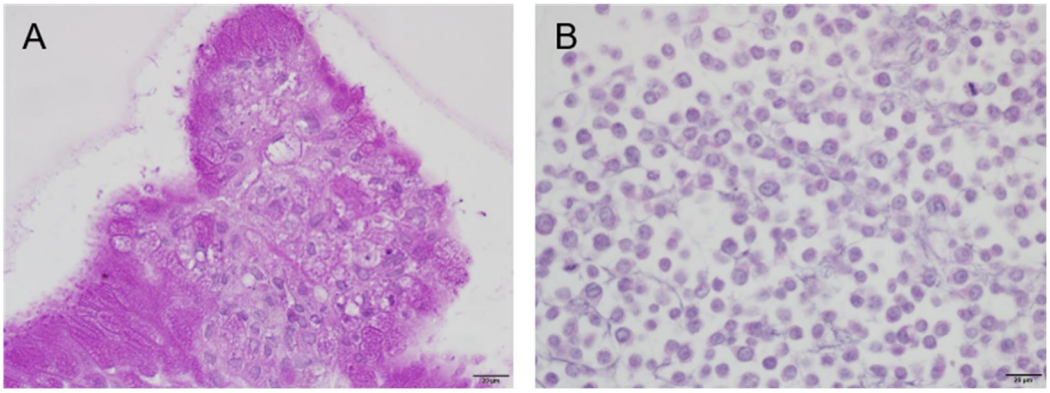

Figure 4. Glycogen accumulation determined by PAS staining in organoids (A) and HepG2 cells (B)

Hepatic protein expression

Protein expression of HNF4α as hepatocyte marker was analyzed by immunofluorescence. Human liver organoids showed positive stain (green) in nuclei whereas the signal of HepG2 was weaker than organoids and seemed non-specific in the cytoplasm.

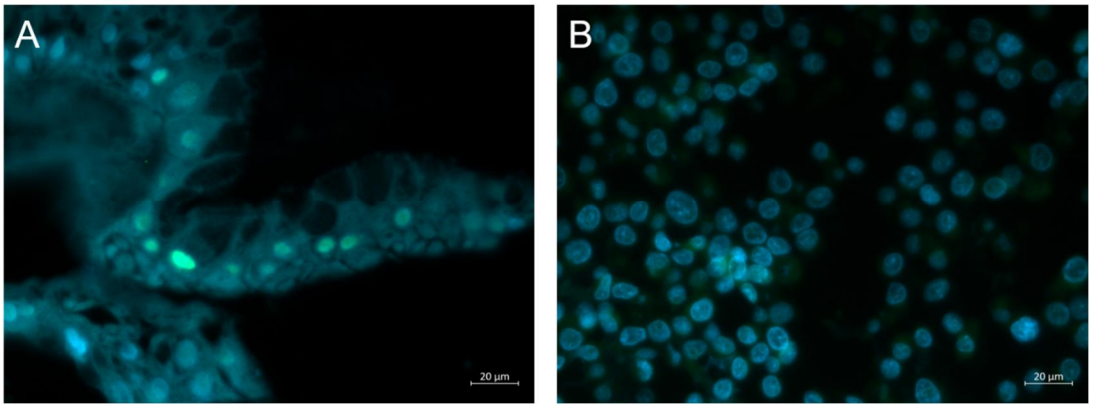

Figure 5. HNF4α expression of organoid (A) and HepG2 cell (B) determined by immunofluorescence (green)

3.2 Viability change caused by PL-inducing drug

Cell viability was shown in Figure 6. 3D human liver organoids survived in high dose PL-inducing drug rather than HepG2 cells for 48 hrs of drug incubation. The viability of HepG2 cell changed drastically and cells even more proliferated than the vehicle control group in amikacin group. Both 2D and 3D cultured hepatocytes died in sertraline, a strong PL-inducing drug. Monolayered HepG2 cells showed obvious cell death at 20 μM of amiodarone and sertraline while the viability of organoids was higher. Acetaminophen was treated as 25 μM or 50 μM and both culture systems didn't show any significant cell viability changes.

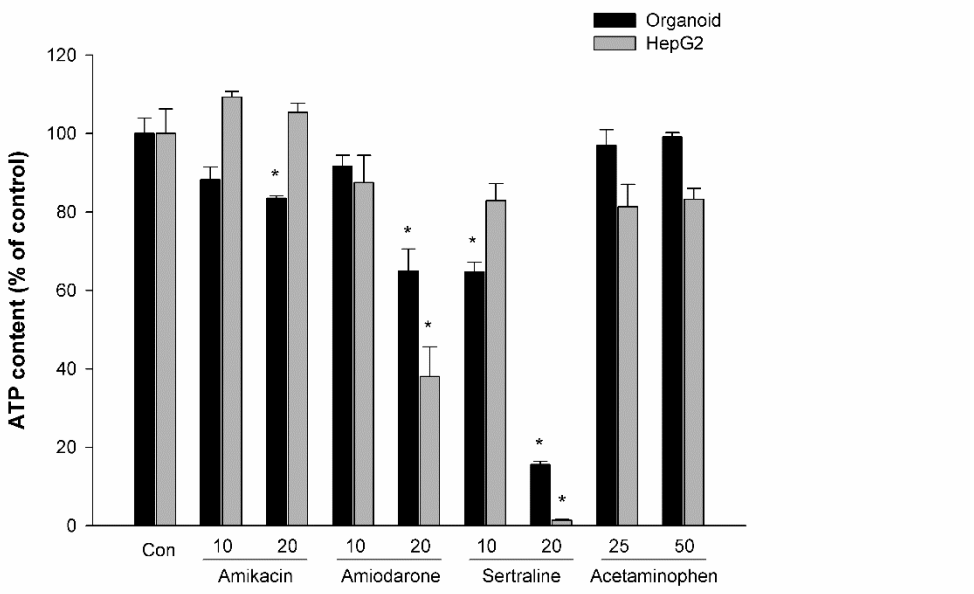

Figure 6. PL-inducing drugs aroused cell death in liver organoid and HepG2 cell. The organoids and HepG2 cells were incubated with each drug (amikacin, amiodarone, sertraline, and acetaminophen) for 48 h, while the group without drug treatment was used as a control. The cell viability was indicated by luminescence of ATP. * indicates values which are significant (p<0.05).

3.3 Albumin content change by PL-inducing drugs

As shown in Figure 7, albumin secretion in HepG2 decreased rapidly depending on the intensity of PL-inducing potential than liver organoids. Both hepatocyte culture systems incubated in 10 μM PL-inducing drugs or 25 μM acetaminophen as a negative control for 48h. Though the mRNA expression levels of organoids and HepG2 cells before incubating PL-inducing drugs were similar, 3D liver organoids kept the capacity of albumin secretion more stable than monolayered HepG2 cells.

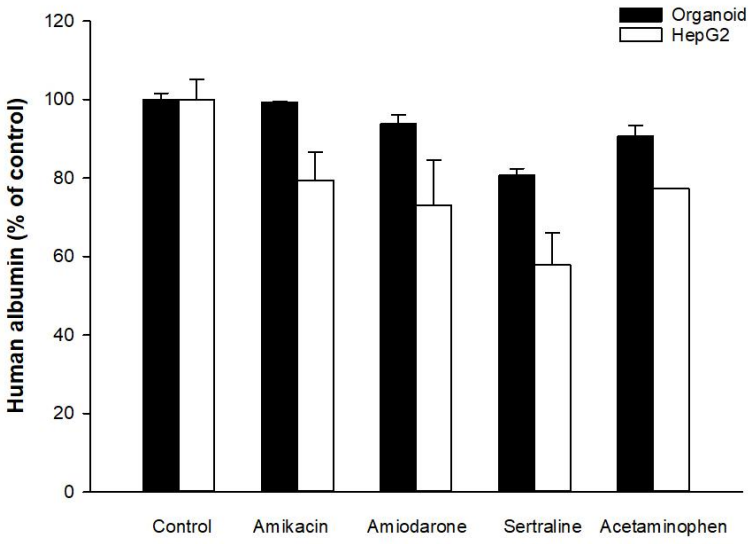

Figure 7. PL-inducing drugs lead to decrease of albumin secretion in liver organoid and HepG2 cells. The organoids and HepG2 cells were incubated with each drug (amikacin, amiodarone, sertraline for 10 μM and acetaminophen for 25 μM) for 48 h, while the group without any drug treatment was used as a control.

3.4 Evaluation of PL

Microscopic evaluation of morphological changes

To determine the morphological changes due to PL, cell slides treated with PL-inducing drug for 48 hr were stained with H&E. The higher possibility of the drug to induce PL, the more cytoplasmic vacuoles was observed.

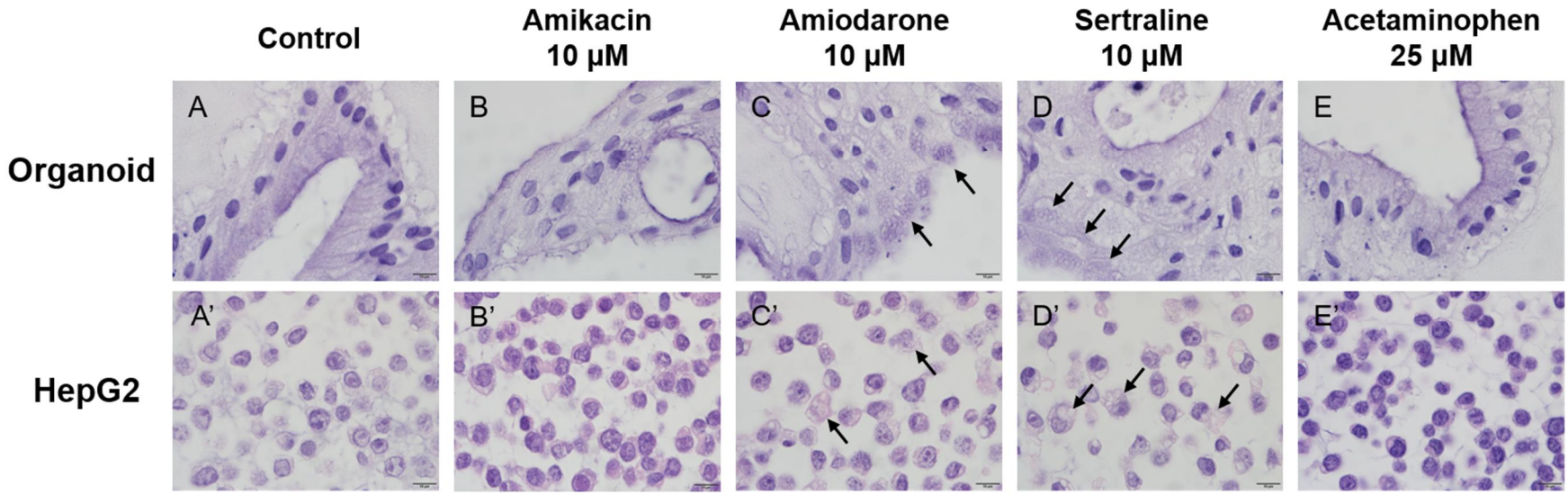

Figure 8. Microscopic evaluation of morphological changes in organoids (A-E) and HepG2 cells (A'-E') caused by PL. Cells were treated with PL-inducing drug for 48 h. Control (A, A'), 10 μM amikacin (B, B'), 10 μM amiodarone (C, C'), 10 μM sertraline (D, D') and 25 μM acetaminophen (E, E'). Arrows indicate PL-induced cytoplasmic vacuolation. All images were taken by 1000 magnification.

Comparison of LAMP-2 expression between two hepatocyte culture systems

Phospholipid accumulation was detected by LAMP-2 immunohistochemistry, as shown in Figure 9. Cells were incubated in each PL-inducing drug for 48 hr. 3D human liver organoid expressed more LAMP-2 positive staining in the cytoplasm than HepG2 cell in the same treatment. Both cell culture systems appear the most strong positive staining located in the cytoplasm in sertraline treatment.

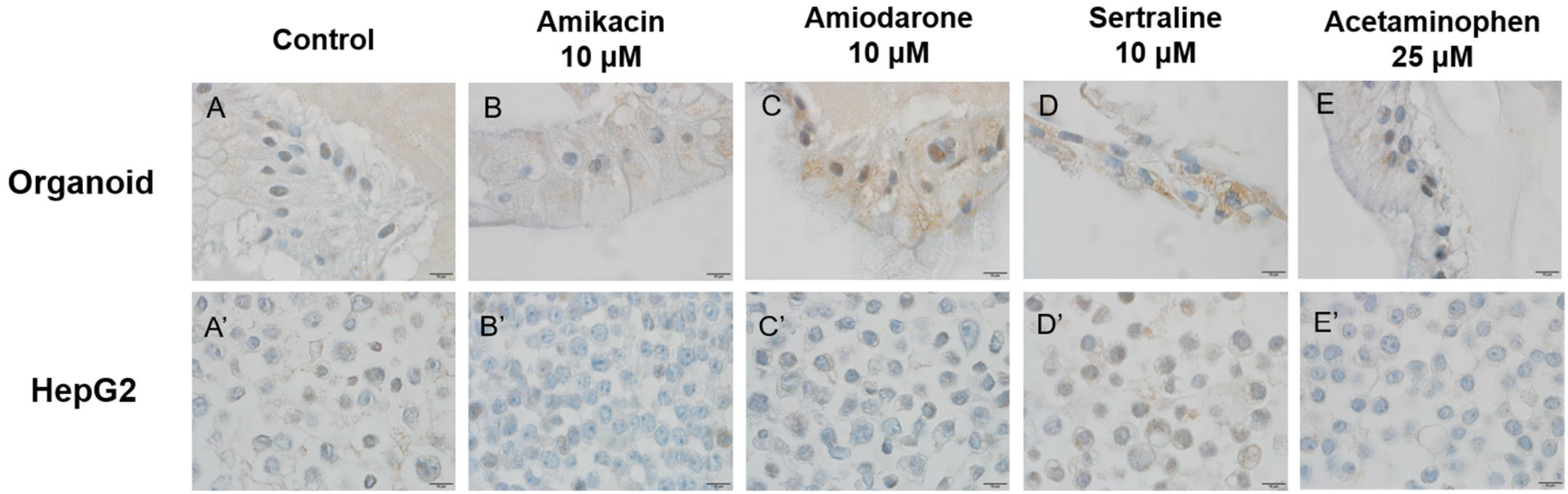

Figure 9. LAMP-2 expression in organoids (A-E) and HepG2 (A'-E') in the condition of PL. Cells were treated with PL-inducing drug for 48 h. Control (A, A'), 10 µM amikacin (B, B'), 10 µM amiodarone (C, C'), 10 µM sertraline (D, D') and 25 µM acetaminophen (E, E'). All images were taken by 1000 magnification.

Transmission electron microscopic examination

The transmission electron microscope was applied to confirm phospholipidosis in organoids (Figure. 10). As shown in Figure 10, the organoids treated with 10 μM amiodarone for 48 hours showed lamellar bodies obviously. In addition, the cell to cell contact of organoids and bile canaliculi were observed.

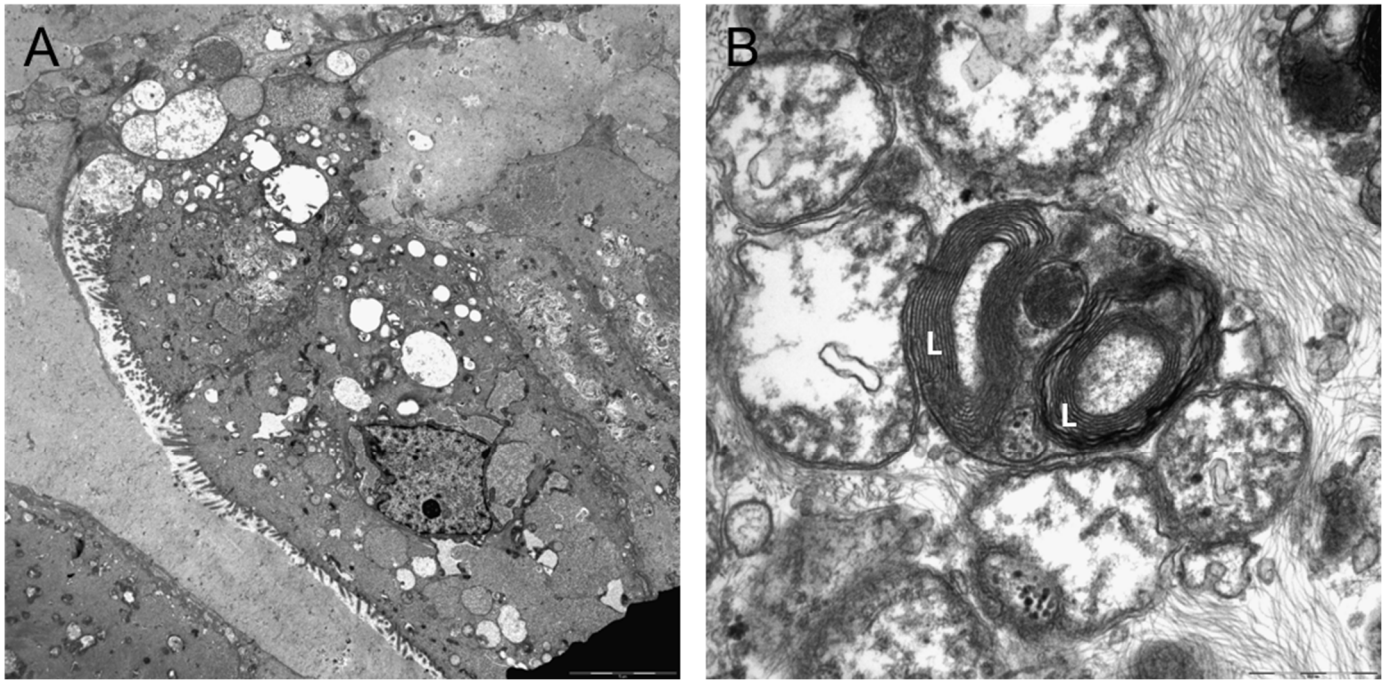

Figure 10. Ultrastructure characteristics of human liver organoids. Cells were differentiated for 12 days. Control (A), 10 μM amiodarone treatment for 48 h (B). Lamellar bodies were shown as letter L.

Gene expression markers for phospholipidosis

To evaluate the severity of drug-induced PL as a toxicogenomic method, fold change value of several gene markers indicating drug-induced PL were measured by qPCR. Cells were treated with amiodarone (5 μM, 10 μM) and sertraline (5 μM, 10 μM) for 48 hours. HepG2 cells incubated with 10 μM sertraline almost died for 48 hours so RNA extraction was not possible. That mRNA expression level of LSS and P8 were upregulated and SLC2A3 was downregulated by PL-inducing drugs was reported previously (Sawada et al., 2004).

Table 3. Fold changes of gene expression level for PL in organoids and HepG2

	LSS	*P8*	*SLC2A3*
Organoid			
Control	1.0023	0.7607	1.0058
Amiodarone 5 μM	1.8986	2.0214	4.6401
Amiodarone 10 μM	1.8830	1.1087	1.1501
Sertraline 5 μM	5.0986	10.2421	4.6163
Sertraline 10 μM	8.0367	11.0668	3.4911
HepG2			
Control	1.0035	1.0377	1.0713
Amiodarone 5 μM	0.5056	0.3833	0.7051
Amiodarone 10 μM	1.0046	3.2357	1.0984
Sertraline 5 μM	1.0066	0.5674	0.5755

4 Discussion

PL caused by drugs in human can be a problem in the pharmaceutical development because there are fatal genetic disorders associated with PL in human, damage to cell wall intensity can lead to cell death, and other cardiac, renal and pulmonary toxicities have been known. Even though PL is found quite frequently as histological findings during exploratory preclinical *in vivo* studies, it is not certain that PL in an animal is toxicologically relevant to human due to the metabolic differences between species. Therefore, it is desirable to identify those compounds with the potential to induce PL in human in the early discovery stage (Chatman et al., 2009). To screen out chemicals which provoke PL in human, cell-based *in vitro* assays using various types of cells from human liver origin have been reported.

We applied robust 3D human liver organoids to predict the potential of drug inducing PL in human. Systemical examination for cellular alterations including viability change, albumin secretion, morphological change, protein expression by special immunostaining, ultra-microstructure by transmission electron microscope examination and gene expression change was proceeded to specialize PL.

As a result of various evaluation about characteristics of the organoids and HepG2, organoids that underwent differentiation to hepatocytes showed more similar nature of hepatocyte than HepG2 cell. The mRNA expression level of CYP3A4 and albumin in organoids and HepG2 cells was marginal comparing with human normal liver tissue. Organoids expressed HNF4α, a hepatic transcription factor, stronger than HepG2 cells in immunofluorescence. In PAS staining, organoids clearly showed glycogen accumulation whereas HepG2 cell rarely stained positively.

Impacts of drug-induced PL to both hepatocyte culture systems were evaluated. As shown in Figure 6, 3D human liver organoids survived in high dose PL-inducing drug rather than HepG2 cells for 48 h of drug incubation. The viability of HepG2 cell changed drastically and the number of cells in amikacin group even increased than the vehicle control group. Both 2D and 3D cultured hepatocytes died at 20 μM of amiodarone and sertraline while the viability of organoids was higher than that of HepG2 cells. This survival change of HepG2 cells seems to be caused by the 2D-cultured condition with a wide exposure area to the drug. Since cells in

in vivo environments interact forming close cellular junctions with surrounding cells, it is considered that the viability changes in organoids that showed resistant to the drug are more likely to reflect the *in vivo* environment. By similar reason, 3D liver organoids kept the capacity of albumin secretion more stable than monolayered HepG2 cells. In the morphological evaluation of PL, the more potent PL-inducing drug administrated, the more cytoplasmic vacuoles were observed in organoids and HepG2 cells. Although these foamy changes are presumed to be a result of drug-induced PL, these vacuolated appearances caused by PL resembles lipid accumulation or artifacts made during processing on light microscopy. To clearly distinguish the induction of PL, LAMP-2, a special marker for lysosome membranes which make it capable to separate PL from lipid droplet, expression was investigated by immunohistochemistry. Comparing the two cell systems stained under the same conditions, the expression of LAMP-2 in organoids seemed more apparent than that in HepG2 cells. To confirm the drug-induced PL in organoids, the differentiated organoids with/without 48-hour 10 μM amiodarone treatment were examined under the transmission electron microscope. PL-characteristic lamellar bodies were observed in only in the amiodarone-treated organoids. In addition, the cell to cell contact of organoids and bile canaliculi were shown in the ultra-microstructure. In the results of the measurement of the mRNA gene expression fold changes, *LSS* and *P8* genes in organoids observed up-regulated as similar with those of the previously known studies (Nioi et al., 2007; Sawada et al., 2004). The mRNA expression changes related PL induction except *SLA2A3* gene were not reproduced in HepG2 as the existing studies.

Our present study showed that 3D cultured human liver organoids were more sensitive to drug-induced PL and less affected by cell death toxicity than HepG2 grown in a monolayer. Since PL is chronic cellular alteration rather than cytotoxic acute toxicity, these results support well that 3D cultured organoids reflected the metabolite-mediated hepatotoxicity *in vivo* (C Shen, Meng, Zhang, & Hu, 2008; Chong Shen, Zhang, Qiu, & Meng, 2006). It has been reported that the detectable toxic concentration (25 μM) of tetracycline in gel entrapped 3D cultured rat hepatocytes treated for 96 hr was very close to the toxic serum concentration in rats (27 μM) (Kikkawa et al., 2006). In contrast, HepG2 cells indicated a more obvious decrease of viability and loss of liver functions which is not corresponding to *in vivo*

environment. 2D cultured HepG2 cells showed changes due to *in vitro* culture condition that exposes cells to drug excessively. The higher sensitivity of organoids to drug-induced PL might mean organoids present phospholipid metabolic disorder more relevantly to *in vivo* situation.

It is difficult to predict drug-induced hepatotoxicity, especially chronic hepatotoxicity like PL, by *in vitro* systems because of the intrinsic limitations of cells outside the body (Peter, Chan, & Silber, 2004). Actually, organoids are composed with a single type of cells existing in the liver so it is not perfect cell system that can accurately predict drug responses *in vivo*. An appropriate cell system *in vitro* for toxicity screening is still very necessary for the early prediction of drug toxicity *in vivo*.

In this study, the drug-induced PL observed in human could be reproduced in primary human hepatocytes in 3D cultured not only in traditional monolayer culture. These differential performances of hepatocyte culture systems may be owing to the different culturing structure and characteristics of cell originated. Conclusively, 3D human liver organoids appeared to be a relevant system for evaluating phospholipidogenic effects of compounds. As cell-based approaches, 3D human liver organoids can be used early in the drug development process to identify chemicals with the potential to induce PL.

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국문 초록

약물에 의해 유도되는 인지질증은 세포 내 리소좀(lysosome)에 인지질-약물 복합체가 형성되어 축적되는 일종의 저장장애이다. 인지질증은 신약개발 과정의 전임상 시험에서 세포질 공포와 같은 형태로 빈번하게 관찰되는 결과이지만, 인지질분해효소 등의 종간 차이 때문에 동물실험에서 관찰된 변화가 사람에서 동일하게 재현되는지 예측하는 것은 어렵다. 따라서 약물이 사람에서 인지질증을 유도하는지 선별하기 위해 사람 유래 간암세포주나 일차(primary) 세포를 이용한 다양한 세포 기반 실험이 보고되고 있다. 오가노이드(organoid)는 사람 성체 조직 유래 세포를 시험관 내 환경에서 팽창시킬 수 있는 3차원 형태의 독특한 세포 배양 시스템이다. 본 연구에서는 Broutier 등 (2016)의 방법에 따라 사람 간 조직 유래 오가노이드를 구축하였고, 오가노이드가 갖는 간세포 특성을 확인한 후 사람에서 인지질증을 유발한다고 알려진 약물을 처리하여 나타나는 변화를 기존의 2차원 형태로 배양된 사람 유래 간암세포주(HepG2)와 비교하였다. 인지질증을 유발하는 약물로는 유발 강도가 다른 amikacin, amiodarone, sertraline을 선정하였고 인지질증 음성 대조군 약물로 acetaminophen을 사용하였다. 약물들을 5 μM 또는 10 μM 농도로 48시간 동안 세포에 처리한 후 오가노이드와 HepG2 세포의 생존성, 알부민 분비량 변화를 분석하고, 형태학적 변화. 조직면역화학염색을 통한 LAMP-2 발현 및 약물에 의한 유전자 발현 변화를 통해 인지질증의 유발을 확인하였고 투과전자현미경 검사를 통해 확진하였다. 오가노이드에서의 CYP3A4 유전자 발현, HNF4α 발현 정도가 HepG2 세포보다 높게 나타났고 오가노이드가 글리코겐을 축적할 수 있다는 것을 확인하였다. 약물에 의한 생존성과 알부민 분비량은 동일 약물, 동일 농도 처리군을 비교하였을 때 오가노이드가 HepG2 세포보다 덜 민감하게 변화하였다. 인지질증 유발 정도는 같은 세기의 약물 처리 시 오가노이드가 HepG2 세포보다 더 민감하게 반응하였고 HepG2 세포는 강력한 인지질증 유발 약물에서는 세포가 사멸하였다. 사람 간 오가노이드는 간암 유래 세포주인 HepG2 세포보다 사람 정상 간세포와 더 유사한 특성을 나타냈고, 3차원 형태로 배양된 사람 간 오가노이드는 인지질증에 의한 독성 변화와 관련하여 기존의 방식으로 2차원적으로 배양된 HepG2 세포보다 생체 내 환경을 더 반영하는 것으로 생각된다. 결론적으로, 사람 간 오가노이드

는 사람에서 약물에 의한 인지질증을 평가하는 데 유용하게 쓰일 수 있다.

핵심어: 인지질증, 3D, 사람 간 오가노이드, HepG2