Short Communication

Long-Term Stability of Cryopreserved Human Hepatocytes: Evaluation of Phase I and II Drug-Metabolizing Enzyme Activities and CYP3A4/5 Induction for More than a Decade

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ABSTRACT

We evaluated the long-term stability of hepatocytes stored in the vapor phase of liquid nitrogen for their viability, cytochrome P450 (CYP) 1A2 activity, CYP3A4/5 activity, uridine diphosphate-glucuronosyl transferase (UGT) activity, sulfotransferase (SULT) activity, and CYP3A4/5 induction during 14 years of preservation. No substantial degradation of viability, CYP1A2 activity, UGT activity, or CYP3A4/5 induction was observed. CYP3A4/5 activity showed a slight decrease after

7 years of storage, and SULT activity gradually decreased during storage, although substantial activities remained even after 14 years. These results indicate that cryopreserved human hepatocytes can be stored stably for more than a decade with little or no change in viability, activity of drug-metabolizing enzymes, or CYP3A4/5 induction, and can be widely applicable to qualitative research in drug metabolism.

Introduction

The metabolic enzymes in the liver are important in the excretion of drugs, toxic substances, and other xenobiotics from the body (Gonzalez, 1988). Understanding the metabolism of a drug candidate is important for estimating individual differences in clinical efficacy or potential adverse effects since many of the differences are caused by individual differences in specific pathways or activities in the metabolism of drug candidates. Wide species differences have been reported in the characteristics of metabolic enzymes (Martignoni et al., 2006), and thus enzymes prepared using human material should be used to evaluate metabolism in humans. Primary hepatocytes provide a complete set of phase I and II drug-metabolizing enzymes, as well as several transporters and cofactors, and are therefore a highly attractive tool for evaluating metabolism, active transport, and enzyme induction (Soars et al., 2007). Cryopreserved primary hepatocytes are commercially available at any time on demand, but their stability during storage is certified for only up to 6 months (Li et al., 1999; Rialland et al., 2000). In this study, we used only limited lots of hepatocytes owing to the limited availability of cryopreserved hepatocytes at the starting point of the study. We confirmed the stability of the viability, metabolic enzyme activity, and CYP3A4/5 induction of cryopreserved human hepatocytes stored for 14 years after preparation.

Materials and Methods

Cryopreserved Human Hepatocytes. All cryopreserved hepatocytes were purchased from Bioreclamation IVT (Baltimore, MD). Lot no. 69 was prepared in 1998 from a 69-year-old Caucasian female, lot no. 77 was prepared in 1998 from a 58-year-old Caucasian male, and lot no. 88 was prepared in 1998 from an 84-year-old Caucasian female. Lot no. 69 and lot no. 77 were used for enzyme activity analysis, and lot no. 88 was used for CYP3A4/5 induction assay.

Reagents. Dulbecco's modified Eagle's medium (DMEM), Leibovitz's L-15 medium, 200 mM glutamine and fetal bovine serum (FBS) were obtained from Thermo-Fisher Scientific (Carlsbad, CA). Long Term Culture Medium was obtained from Biopredic International (Saint Grégoire, France). All other reagents and chemicals were of the highest quality obtainable from commercial sources.

Thawing and Seeding of Cryopreserved Human Hepatocytes. For the enzyme activity analysis, cryopreserved hepatocytes were thawed and suspended in DMEM using a previously reported procedure (Li et al., 1999) with minor modifications. The hepatocyte suspension was seeded at a density of 3.0×10^5 viable cells/1 ml per well in 12-well collagen-coated plates.

For the induction study, hepatocytes were thawed in a water bath at 37°C and suspended in Leibovitz's L-15 medium containing 10% FBS and 0.5 g/liter glucose. The hepatocyte suspension was centrifuged at 55g for 3 minutes, and the hepatocytes were resuspended in Long Term Culture Medium containing 10% FBS. The hepatocyte suspension was seeded at a density of 6.0×10^5 viable cells/1 ml per well in 12-well collagen-coated plates.

Measurement of Viability. Trypan blue (0.15% to 0.2%) was added to the hepatocyte suspension, and the viability of the cells was determined as the percentage of cells, excluding the dye compared with the total cell number.

Measurement of CYP3A4/5 Activity. Testosterone 6 β -hydroxylation activity was measured to determine the CYP3A4/5 activity. Testosterone (50 μ M) was added to each hepatocyte suspension and incubated for 1 hour under 5% CO₂ at 37°C. After incubation, the hepatocyte suspension was extracted by ethyl acetate and the extracted fraction was dried and resuspended in methanol/ultrapure water (1:1). This solution was subjected to high-performance liquid chromatography (HPLC) with a TSKgel ODS120T (5 μ m, 250 $\times$ 4.6 mm i.d.; Tosoh Corporation, Tokyo, Japan) and a UV detector (254 nm). All experiments were performed in duplicate.

Measurement of 7-Ethoxycoumarin Metabolic Activity. The metabolic activity of 7-ethoxycoumarin (7-EC) was evaluated as a marker for CYP1A2 activity, and 7-hydroxycoumarin glucuronide (7-HCG) and 7-hydroxycoumarin sulfate (7-HCS) formation through 7-hydroxycoumarin (7-HC) were analyzed to determine glucuronidation and sulfation activity, respectively. 7-EC (75 μ M) was added to the hepatocyte suspension and incubated for 4 hours under 5% CO₂ at 37°C. The reaction was terminated by freezing at -30°C. After thawing, the

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ABBREVIATIONS: CYP, cytochrome P450; DMEM, Dulbecco's Modified Eagle medium; 7-EC, 7-ethoxycoumarin; FBS, fetal bovine serum; 7-HC, 7-hydroxycoumarin; 7-HCG, 7-hydroxycoumarin glucuronide; 7-HCS, 7-hydroxycoumarin sulfate; HPLC, high-performance liquid chromatography; iPSC-HHs, inducible pluripotent stem cells into human hepatocyte-like cells; SULT, sulfotransferase; UGT, uridine diphosphate glucuronosyltransferase.

Fig. 1. Annual changes in viability of human hepatocytes. Viability was determined by the trypan blue exclusion method. The viability of lot no. 69 and lot no. 77 was determined based on a single result for each storage period, and the viability of lot no. 88 was determined based on the mean values of several results (1, 2, 2, 7, 3, 11, 5, 7, 5, 1, and 1 results at years 3, 5, 7, 8, 9, 10, 11, 12, 13, 14, and 16 years, respectively). The data for each time point are expressed as the mean values of eight separate counts, performed using hemocytometers. The viability of lot no. 69 is shown with closed circles, lot no. 77 with open circles, and lot no. 88 with open triangles.

hepatocyte suspension was centrifuged at 1500g for 10 minutes, and the supernatant was subjected to HPLC with a SUPERCOSIL LC-8 (5 μ m, 50 $\times$ 4.6 mm i.d.; Sigma-Aldrich) and a UV detector (325 nm) (Hiller and Cole, 1995). Each study was performed in duplicate.

Evaluation of CYP3A4/5 Induction Activity. The hepatocytes were cultured with FBS for 1 day to induce attachment to the plates and then for 3 days without FBS for stabilization in Long Term Culture Medium under 5% CO₂ at 37°C. Then, the hepatocytes were treated with Long Term Culture Medium containing rifampin (10 μ M) for 3 days. The rifampin-containing media was changed every day. After treatment with rifampin, the hepatocytes were rinsed with fresh media and incubated with media containing testosterone (250 μ M) for 2 hours, and the formation of 6 β -hydroxytestosterone was analyzed by HPLC as described in the *Measurement of CYP3A4/5 Activity* section).

Results and Discussion

Human hepatocytes are widely used to evaluate drug metabolism, active transport, and enzyme induction (Sahi et al., 2010), and they are also used for evaluation of drug-induced liver injury. HepG2, HepaRG,

Fig. 2. Annual changes in CYP3A4/5 activity of human hepatocytes. CYP3A4/5 activity was measured by testosterone 6 β -hydroxylation activity. The data are expressed as mean values of duplicate incubation results. Colors represent the CYP3A4/5 activity of lot no. 69 (gray) and lot no. 77 (black). ND, not determined.

Fig. 3. Annual changes in 7-EC metabolic activity. The graph shows formation of the total metabolites of 7-EC (7-HC, 7-HCG, and 7-HCS) in lot no. 69 (A) and lot no. 77 (B). The data are expressed as mean values of duplicate incubation results. Colors represent the 7-EC metabolic activity of 7-HC (black), 7-HCG (gray), and 7-HCS (white). ND, not determined.

and inducible pluripotent stem cells into human hepatocyte-like cells (iPSC-HHs) are used to evaluate drug metabolism and hepatotoxicity (Krueger et al., 2014; Saito et al., 2016); however, HepG2 and HepaRG are poor for detection of hepatotoxicity because of their limited function and production of metabolic enzymes. iPSC-HHs would be an ideal research tool, but production of cells possessing complete hepatocyte function is exceedingly difficult, although further progress is expected. Cryopreserved hepatocytes that have been stored stably in the vapor phase of liquid nitrogen can be transported over long distances more easily and can be used at any time the need arises. In this series of studies, we evaluated the stability of human primary hepatocytes during cryopreservation for longer than a decade as a tool for investigation of drug metabolism.

Viability, as evaluated by the trypan blue exclusion method (McLimans et al., 1957), did not change during the 14 years of storage in any of the three lots of hepatocytes (Fig. 1), indicating that the integrity of the cytoplasmic membrane was sustained during storage.

CYP1A2 and CYP3A4/5 are localized in the microsomal membrane (Gonzalez, 1988). A slight decrease in CYP3A4/5 activity was observed after 8 years compared with activity evaluated in the first 7 years, but the residual activity ratio at 14 years, compared with that at 7 years, was about 75% (Fig. 2). 7-EC is metabolized to 7-HC by CYP1A2, and 7-HC is subsequently metabolized to 7-HCG by UGT and to 7-HCS by SULT. The total amount of 7-HC, 7-HCG, and 7-HCS, which indicates the actual 7-HC formation activity, did not decrease after 14 years (Fig. 3).

Fig. 4. Annual changes of glucuronidation and sulfation activity of 7-HC. The graph shows the ratio of 7-HCG formation to 7-HC formation and the ratio of 7-HCS formation to 7-HC formation through 7-HC. The data are expressed as mean values of duplicate incubation results. The UGT activity of lot no. 69 is shown with closed circles and lot no. 77 with open circles. The SULT activity of lot no. 69 is shown with closed triangles and lot no. 77 with open triangles.

Formation of 7-HCG and 7-HCS from 7-HC were analyzed as markers for the activity of UGT and SULT, respectively (Fig. 4). UGTs are located in the microsomal membrane, and SULTs are located in the cell cytoplasm. UGT activity, expressed as the ratio of the 7-HCG concentration to the 7-HC concentration (7-HCG/7-HC), was maintained during the 14 years of preservation (Fig. 4); however, SULT activity, expressed as the ratio of the 7-HCS concentration to the 7-HC concentration (7-HCS/7-HC), showed a clear tendency to decrease (Fig. 4), and this finding was consistent with reports that the SULT activity in rat hepatocytes was decreased by cryopreservation (Utesch et al., 1992).

Fig. 5. Annual changes in CYP3A4/5 induction activity of human hepatocytes. The graph shows CYP3A4/5 activity in hepatocytes treated with 10 μ M rifampin or 0.1% DMSO as a control for 3 days. CYP3A4/5 activity was measured by testosterone 6 β -hydroxylation activity. Testosterone 6 β -hydroxylation activity is expressed as the mean or where error bars are shown as the mean $\pm$ S.D., calculated based on individual data for a number of tests (1, 2, 2, 7, 3, 11, 5, 7, 5, 1, and 1 for years 3, 5, 7, 8, 9, 10, 11, 12, 13, 14, and 16, respectively). The activity after treatment with rifampin is shown with closed circles and the control is shown with open circles.

CYP3A4/5 induction, which is triggered by pregnane X receptor activation (Chai et al., 2013), was not changed during preservation, and CYP3A4/5 activity after rifampin treatment was maintained at a high level throughout preservation (Fig. 5), indicating that DNA to RNA transcription and subsequent protein synthesis were maintained, to deliver a sufficient amount of CYP3A4/5 enzyme.

These results demonstrate the survival of hepatocytes for more than a decade. It should be noted that this study was performed only in limited lots of hepatocytes. This practical information concerning long-term hepatocyte stability should lead to improvements in the use of cryopreserved hepatocytes, which are an excellent tool for qualitative evaluation of drug metabolism and metabolism mediated drug-drug interactions.

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