Cytochrome P450 Expression of Cultured Rat Small Hepatocytes After
Long-Term Cryopreservation

Hidekazu Ooe, Junko Kon, Shigeki Miyamoto, Yoshiyasu Ozone,
Shin-ichi Ninomiya, Toshihiro Mitaka

Department of Pathophysiology, Cancer Research Institute (H.O., J.K.,
T.M.), First Department of Surgery (S.M.), Sapporo Medical University
School of Medicine, Sapporo, Japan; Daiichi Pure Chemical Co. Ltd., Tokai,
Ibaraki, Japan (Y.O., S.N.)
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*All correspondence should be addressed to

Dr. Toshihiro Mitaka, M.D., Ph.D.  Department of Pathophysiology, Cancer Research Institute, Sapporo Medical University School of Medicine, South-1, West-17, Chuo-Ku, Sapporo 060-8556, JAPAN
Tel: +81-11-611-2111 ext. 2390
Fax: +81-11-615-3099
Email: tmitaka@sapmed.ac.jp

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Abbreviations used: DMEM, Dulbecco’s modified Eagle’s medium; DMSO, dimethyl sulfoxide; HPLC, high-performance liquid chromatography; 3MC, 3-methylcholanthrene; MH, mature hepatocyte; PB, phenobarbital; PCN, pregnenolone-16α-carbonitrile; SH, small hepatocyte
Abstract

Small hepatocytes (SHs) are hepatic progenitor cells that can be cryopreserved for a long time. After thawing, the cells can proliferate and, when treated with Matrigel, they can differentiate into mature hepatocytes (MHs). In this study we investigated whether cryopreserved SHs could express cytochrome P450s (CYP), whether CYP expression was induced by appropriate inducers, and whether CYP activities were measurable. 3-Methylcholanthrene (3-MC), phenobarbital (PB), pregnenolone-16α-carbonitrile (PCN), and ethanol (EtOH) were used as inducers for CYP1A, 2B, 3A, and 2E, respectively. Immunoblot analysis indicated that cryopreserved SHs constitutively expressed CYP1A1/2, CYP2E1, and CYP3A2 as much as 26 days after plating. Significant expression of CYP1A1/2 and 3A2 in the cells treated with Matrigel was induced by 3MC and PCN, respectively. Although Matrigel did not upregulate the enzymatic activity of CYP1A, CYP3A and CYP2E activities increased. Induction of CYP1A and CYP3A activities by each inducer was observed in cryopreserved cells treated with Matrigel. Although the expression of CYP2B1 could be detected in subcultured SHs treated with PB, it was not detected in cryopreserved SHs. The activity of NADPH-cytochrome P450 reductase was measured in both subcultured and cryopreserved SHs though the activities in both were about 30% of that of MHs. Profiles of 14C-testosterone metabolites were examined in cultured MHs and in cryopreserved SHs by HPLC. Similar peaks for testosterone metabolites in MHs and SHs were observed in the same elution time. These results indicate that, although induction of CYP3A and 2B in cryopreserved SHs is inferior to that in subcultured ones, SHs can maintain the expression and activities of CYPs after long-term cryopreservation.
Introduction

Cytochrome P450s (CYP) constitute a superfamily of mono-oxygenases that play a key role in either the detoxification or the metabolic activation of xenobiotics (Guengerich et al., 1990; Wrighton et al., 1992; Gonzalez et al., 1994). The CYPs involved in xenobiotic metabolism are concentrated in liver cells. In vivo, many of the constitutive CYPs are actually inducible by xenobiotics such as aromatic hydrocarbons, drugs, alcohol, etc. However, in traditional culture systems, it is very difficult to maintain the CYP activity of hepatocytes (Bissell et al., 1980). After cryopreservation of both rodent and human primary hepatocytes, rapid loss of hepatic differentiated functions, including CYP activity, has been reported in the cultured cells (Jackson et al., 1985, Loretz et al., 1989, Sun et al., 1990, Chesne et al., 1993, de Sousa et al., 1996, Swales et al., 1996, Garcia et al., 2003).

Small hepatocytes (SHs) have been identified as proliferating cells with hepatic characteristics (Mitaka et al., 1992, 1995, Tateno et al., 1996, Hino et al., 1999, Kon et al., 2006). We showed that a single SH could clonally proliferate and form flat colonies (Mitaka et al., 1999). It was also reported that maturation of the proliferating SHs was induced by the application of Engelbreth-Holm-Swarm sarcoma-derived matrix (Matrigel; Sugimoto et al., 2002). Recently, SHs were shown to express several CYP proteins even after more than one month of culture (Miyamoto et al., 2005). In addition, the enzyme activities were induced and measured by the addition of appropriate inducers. On the other hand, we reported that SHs could be cryopreserved for more than 6 months and that, even after thawing, the cells could maintain growth ability and hepatic differentiated functions (Ikeda et al., 2002).

In this study we investigated whether cryopreserved SHs could express CYPs, whether CYP expression was inducible, and whether CYP activities were measurable. The results showed that the protein expression of CYP1A1/2, CYP3A2, and CYP2E1 and the activities of
CYP1A, CYP3A, CYP2E and NADPH-cytochrome P450 reductase could be detected in the cells. Radiolabeled testosterone could be metabolized in long-term cultured cells and radioactive metabolites in cultured mature hepatocytes (MHs) were detected by high-performance liquid chromatography (HPLC).
Materials and Methods

Isolation and Culture of SHs

F344 rats (Sankyo Lab Service, Tokyo, Japan), weighing 170-240 g, were used. All animals received humane care and the experimental protocol was approved by the Committee of Laboratory Animals according to Sapporo Medical University guidelines. Hepatic cells were isolated by the two-step collagenase perfusion method. Details of the isolation and culture procedure for SHs were previously described (Mitaka et al., 1999). Finally, 6 x 10^4 viable cells/cm² were seeded on a 100-mm dish and cultured in Dulbecco’s modified Eagle’s medium (DMEM; SIGMA Chem. Co., St Louis, MO) supplemented with 20 mM HEPES, 25 mM NaHCO₃, 30 mg/L L-proline, 10% fetal bovine serum (FBS, HyClone, Logan, UT), 10 mM nicotinamide (Katayama Chemical Co., Osaka, Japan), 1 mM ascorbic acid 2-phosphate (Wako Pure Chem. Co., Tokyo, Japan), 10 ng/ml epidermal growth factor (EGF; Collaborative Research Inc., Lexington, MA), 0.5 mg/L insulin, 10⁻⁷ M dexamethasone, and antibiotics. After 4 days of culture, 1% dimethyl sulfoxide (DMSO; Aldrich Chem. Co., Milwaukee, WI) was added to the medium.

Subculture and Cryopreservation of Small Hepatocyte Colonies

As previously reported (Mitaka et al., 1999; Ikeda et al., 2002), colonies consisting of 30-50 cells were observed at day 14 after plating. To collect SHs, the colonies were detached from dishes and immediately replated or cryopreserved at −80°C until use (Fig. 1A). SH colonies (3-5 x 10^3 colonies/60-mm dish) were replated on dishes coated with rat tail collagen. One day after replating, the medium was replaced with serum-free modified DMEM supplemented with 1% DMSO. Fourteen days after replating, some dishes were treated with Matrigel (1 mg/dish; Becton Dickinson, Bedford, MA).

Immunoblots for CYP proteins

3-Methylcholanthrene (3-MC; Wako), phenobarbital (PB; Wako),
pregnenolone-16α-carbonitrile (PCN; SIGMA), and ethanol (EtOH; Katayama) were used as inducers for CYP1A, 2B, 3A, and 2E, respectively. Eight days after Matrigel treatment, fresh medium containing the inducer (5 μM 3MC, 2 mM PB, 2 μM PCN, or 100 mM EtOH) was added. To enhance the CYP expression, the medium containing each inducer was renewed every day for 3 consecutive days before harvest (Fig. 1A).

For immunoblots, the dishes were washed with PBS twice and then treated with 1 ml of lysis buffer (10 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 5 μg/ml leupeptin, 5 μg/ml pepstatin A) for 1 hr at 4°C. The cells were scraped and used for protein extraction. Samples (15 μg/lane) were separated by SDS-PAGE. Rabbit anti-CYP1A2, anti-CYP3A2, goat anti-CYP2B1, and anti-CYP2E1 (Daiichi Pure Chem. Co, Tokyo, Japan) antibodies were used for immunoblots. The details of the method were previously described (Miyamoto et al., 2005).

**Enzyme activity of CYPs**

For the measurement of CYP enzyme activity, the cells were washed with KH buffer (0.96% Krebs-Henseleit buffer powder, 2.5 mM CaCl₂・2H₂O, 25 mM NaHCO₃ [pH 7.5]) twice at 37°C. After addition of 1.5 ml of KH buffer containing the substrates, 3 μM ethoxyresorufin, 125 μM testosterone, or 300 μM chlorzoxazone, the cells were incubated for 1 hr at 37°C. The reaction reagents were collected and centrifuged at 12,000 x g for 10 min at 4°C. Then the supernatants were collected and dosed with 250 μl of methanol containing 10 μg/ml phenacetin to stop the reaction. Samples were kept at -80°C until use. Metabolites catalyzed by each CYP were fluorometrically measured according to the method of Burke and Mayer (Burke et al., 1974) with some modifications (Miyamoto et al., 2005). CYP activities were determined by 7-ethoxyresorufin-O-deethylation (CYP1A), testosterone-6β-hydroxylation (CYP3A), and chlorzoxazone 6-hydroxylation (CYP2E).

**NADPH-cytochrome P450 reductase activity**
Activity of NADPH-cytochrome P450 reductase was measured by the method of Yukawa et al. (1983).

**Profiles of \(^{14}\)C-testosterone metabolites**

SHs treated with Matrigel at day 22 and cultured MHs at day 1 were examined. MHs were seeded on a 100-mm dish and cultured in DMEM containing 10% FBS. The cells were washed with KH buffer twice at 37°C. After addition of 1.5 ml of KH buffer containing 125 \(\mu\)M \(^{14}\)C-testosterone (Amersham Biosciences Co., Piscataway, NJ) to the dish, the cells were incubated for 1 hr at 37°C. The reaction reagents were filtered by centrifugation (5,000 x g, 5 min, 4°C) with an UltraFree-CL (0.45 \(\mu\)m; Millipore, Billerica, MA) and 100 \(\mu\)l was analyzed. Metabolites were detected using HPLC (LC-10ADvp; Shimadzu, Kyoto, Japan) with a Cosmosil 5C18-AR column (4.6 x 250 mm; Nacalai Tesque, Kyoto, Japan) and a radioactivity detector (FLO-ONE 525TR; Packard Instruments, Meriden, CT). The column temperature was set at 40°C and the UV-detector was set at 240 nm. The mobile phase was water/tetrahydrofuran (5:1, v/v) as solvent A and methanol as solvent B. Gradient conditions were 0-20 min, 20-30% B (linear gradient); 20-20.5 min, 30-70% B (linear gradient); 20.5-24 min, 70% B; 24-25 min, 70-20% B (linear gradient); 25-30 min, 20% B. The flow rate was 1 ml/min.

**Statistical Analysis**

Statistical analysis was performed using Tukey’s honestly significant difference test. A \(P\)-value of <0.05 was considered significant.
Results and Discussion

SHs began dividing from day 3 and rapidly proliferated to form colonies. At day 14 after plating many colonies consisting of 30-50 cells were detached from dishes. The colonies were replated on new dishes or cryopreserved for more than one month. Most subcultured colonies could attach but only about 60% of thawed colonies could attach to collagen-coated dishes (Ikeda et al., 2002). Attached cells proliferated to form a large monolayer colony (Fig. 1B-b). Other types of cells such as liver epithelial cells and stellate cells also survived cryopreservation but were few in number. As previously reported (Sugimoto et al., 2002), when SH colonies were treated with Matrigel, the shape of proliferating SHs changed from flat to rising/piled-up and size from small to large. The alteration resulted in the differentiation of SHs into MHs. The cells could express not only tryptophan 2,3-dioxygenase and serine dehydratase but also liver-enriched transcription factors such as hepatocyte nuclear factor (HNF) 4α, HNF6, CCAAT/enhancer binding protein α (C/EBP α), and C/EBPβ, which are known to be restrictedly expressed in highly differentiated hepatocytes (Sugimoto et al., 2002). Expression of CYP protein and activity was also demonstrated in the SHs treated with Matrigel (Miyamoto et al., 2005). Therefore, we first examined the effect of Matrigel treatment on the morphology of cryopreserved SHs. As shown in Fig. 1B-c, the shape of most cells in colonies changed from flat to rising/piling-up within one week. This result was coincident with that of subcultured SHs (Miyamoto et al., 2005). We then examined the expression of CYP proteins by immunoblotting. As shown in Figures 2A and 2B, CYP1A1/2 and CYP3A2 were constitutively expressed and not induced by Matrigel in both subcultured and cryopreserved SHs. Expression of CYP2B1 was detected in subcultured SHs treated with PB but not in cryopreserved SHs. On the other hand, CYP2E1 was detected in subcultured SHs treated with Matrigel but constitutively expressed in cryopreserved ones. When SHs were treated with 3MC and PCN for 4 days,
CYP1A1/2 and CYP3A2 were induced in the cells with Matrigel, respectively. However, this induction was not observed in the cells without Matrigel. As shown in Figure 2B, the pattern of CYP1A1/2 and CYP3A2 induction showed similarity between subcultured (3.6-fold and 6.9-fold) and cryopreserved (2.4-fold and 2.4-fold) SHs. CYP3A1 is known to be the dominant 3A after the induction. The cross-reactivity of the antibody between 3A1 and 3A2 is not clear. Therefore, the induced 3A may be 3A1 in the present experiment.

Induction of CYP2E1 by EtOH was not detected in either subcultured or cryopreserved SHs.

Next we investigated whether cryopreserved SHs could have CYP enzymatic activities. As shown in Figure 2C, activities of 7-ethoxyresorufin-O-deethylation (CYP1A), testosterone-6β-hydroxylation (CYP3A), chlorzoxazone 6-hydroxylation (CYP2E), and testosterone 16β-hydroxylation (CYP2B) were measured in cryopreserved SHs. In the cells without Matrigel the activities of CYP1A and CYP3A were quite low and that of CYP2B was not detected. Enzyme activities of CYP2E and CYP3A were induced in the cells with Matrigel (2.0-fold and 8.7-fold, respectively). When SHs were treated with inducers, induction of CYP1A activity by 3MC was observed (32-fold without Matrigel and 23-fold with Matrigel). Induction of CYP3A activity was observed in the cells with Matrigel (1.9-fold), whereas SHs without Matrigel did not show induction of CYP3A. In spite of EtOH treatment CYP2E activity did not show any significant difference between the cells with and without Matrigel. Very low activity of CYP2B was observed only in the cells with both Matrigel and PB (data not shown). There was a close relationship between the protein expression and the enzymatic activity of each CYP, although some discrepancies were found; CYP3A2 protein was not adequately induced in the cells with Matrigel and the CYP1A activity was low although the protein was expressed. To investigate the causes of these discrepancies, we examined the activity of NADPH-cytochrome P450 reductase in SHs. As shown in Figure 2C, the activity was observed in both subcultured and cryopreserved SHs,
although measured activity was about 0.3-fold that of MHs. The results indicated that the reductase activity might affect CYP activity.

Although we did not detect expression and activity of CYP2B in the cryopreserved SHs, the present experiment and a previous one (Miyamoto et al., 2005) showed that subcultured SHs could express it. To determine the reason, the expression of CYP2B1 in cryopreserved SHs was measured during culture. At the time of thawing, the cells possessed the protein but the expression rapidly decreased with time in culture. Within 3 days the enzyme was not detected in the cells (data not shown). Further experiments are necessary to clarify the mechanism of the loss.

Next we investigated the profiles of testosterone metabolites in SHs treated with Matrigel. As shown in Figure 3, several metabolites of testosterone were indicated as peaks in both MHs and SHs; for example, 6β-hydroxytestosterone, 16α-hydroxytestosterone, and 16β-hydroxytestosterone. Although the heights of the peaks were relatively lower in SHs than in cultured MHs and some metabolites were not detected in SHs, testosterone could be sequentially metabolized in the cryopreserved SHs as effectively as in cultured MHs. The quite low peak of 16β-hydroxytestosterone in SHs was due to the low expression of CYP2B. The results correlated with those of both protein and enzyme activity experiments.

In the present study we showed that cryopreserved SHs could maintain the inducibility of both CYP proteins and their enzyme activities. Until now, to supply hepatocytes used for pharmacological and pharmaceutical investigations, it has been necessary to isolate MHs for every experiment because there are few cell lines possessing hepatic differentiated functions, especially CYP enzyme activities. In addition, the number of obtainable cells depends on the number of cells in the individual because a method for proliferating hepatocytes with differentiated functions has not been established. However, by using SHs, these problems may be resolved because SHs can be isolated from adult rodents, continue proliferating, and
can be cryopreserved for a long time. Whenever hepatocytes are required, cryopreserved SHs may be thawed and plated on dishes. After the SHs proliferate and reach the required number, maturation of the cells is easily induced by Matrigel. Although improvements of the culture conditions are necessary, e.g., an increase of CYP2B expression, we think that cryopreserved SHs may be very useful for pharmacological and toxicological studies.
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References


Footnotes

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Reprint requests should be addressed to

Dr. Toshihiro Mitaka, M.D., Ph.D. Department of Pathophysiology, Cancer Research Institute, Sapporo Medical University School of Medicine, South-1, West-17, Chuo-Ku, Sapporo 060-8556, JAPAN
Tel: +81-11-611-2111 ext. 2390
Fax: +81-11-615-3099
Email: tmitaka@sapmed.ac.jp
Legends for Figures

Figure 1

(A) The experimental schedule. Small hepatocytes (SHs) were isolated from a rat liver and cultured in modified DMEM. SH colonies were detached from the culture dishes at 14 days after plating and immediately replated or cryopreserved at -80°C for more than one month. One day after replating, the medium was replaced with serum-free DMEM containing 1% DMSO. Fourteen days later, the cells were overlaid with Matrigel. Arrows indicate the timing of the CYP inducer treatment. Cells at 26 days were harvested from dishes and examined for CYP expression and enzyme activities. (B) Phase-contrast micrographs of cryopreserved SHs at days 2 (a) and 21 (b). At day 7 after Matrigel treatment most cells in colonies were piled-up (c). All photos are the same magnification. Scale bar, 300 µm.

Figure 2

(A) Immunoblots for CYP proteins induced by various CYP inducers in subcultured SHs and cryopreserved SHs treated with or without Matrigel. 3-MC, PB, EtOH and PCN were used for CYP1A1/2, CYP2B1, CYP2E1 and CYP3A2, respectively. Samples (15 µg/lane) were separated by 10% SDS-PAGE. Relative expression of each CYP was estimated from immunoblots and is presented in (B). (C) CYP enzymes and NADPH-cytochrome P450 reductase activity. Activities of CYP proteins were induced by various inducers in cryopreserved SHs treated with or without Matrigel. The cells were exposed to KH buffer containing the substrate, 3 µM ethoxyresorufin (CYP1A), 125 µM testosterone (CYP3A), or 300 µM chlorzoxazone (CYP2E) for 1 hr at 37°C and reaction reagent was collected. Resorufin, 6β-hydroxytestosterone, and 6-hydroxychlorzoxazone were detected as metabolites. Activities of CYPs (pmol/min/dish) were calculated. Activity of NADPH-cytochrome P450 reductase was measured in MHs, subcultured SHs, and
cryopreserved SHs. Protein (0.1 mg) was added to buffer (1 ml) containing 0.1 M Tris-HCl (pH 7.5), 25 µM cytochrome c, and 0.1 mM NADPH. Reduction of cytochrome c was detected with a spectrophotometer (550 nm) and activity was estimated as unit/mg (mM/min/mg protein). “N” indicates no protein.

Excluding reductase activity of MHs, all data are means ± SD from three different experiments. Asterisks indicate significant induction with the inducer or Matrigel (*; p<0.05, **; p<0.01, ***; p<0.001).

Figure 3

Profiles of 14C-testosterone metabolites in SHs and MHs. Cultured SHs at day 22 and MHs at day 1 were incubated in KH buffer containing 125 µM 14C-testosterone for 1 hr at 37°C. The reaction reagents were analyzed using HPLC. The main metabolites of testosterone catalyzed by CYPs are indicated by arrows.
The table shows the expression levels of various genes in subcultured and cryopreserved SHs.

<table>
<thead>
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<th>Gene</th>
<th>Subcultured SHs</th>
<th>Cryopreserved SHs</th>
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<tr>
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<tr>
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<tr>
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