ABSTRACT:

Cytochromes P450 (P450) constitute a superfamily of monoxygenases that play a key role in either the detoxification or the metabolic activation of xenobiotics (Guengerich and MacDonald, 1990; Wrighton et al., 1992; Gonzalez and Gelboin, 1994). The P450s involved in xenobiotic metabolism are concentrated in liver cells. In vivo, many of the constitutive P450s are actually inducible by xenobiotics such as aromatic hydrocarbons, drugs, alcohol, etc. However, in traditional culture systems, it is very difficult to maintain the P450 activity of hepatocytes (Bissell and Guzelian, 1980). After cryopreservation of both rodent and human primary hepatocytes, rapid loss of hepatic differentiated functions, including P450 activity, has been observed. Moreover, it is very difficult to maintain the P450 activity of cryopreserved cells. Therefore, it is very difficult to maintain the P450 activity of cryopreserved cells and to maintain the expression and activities of P450s after long-term cryopreservation.

ABBRVIATIONS: P450, cytochrome P450; DMEM, Dulbecco’s modified Eagle’s medium; DMSO, dimethyl sulfoxide; EtOH, ethanol; HPLC, high-performance liquid chromatography; KH, Krebs-Henseleit; 3-MC, 3-methylcholanthrene; MH, mature hepatocyte; PB, phenobarbital; PCN, pregnenolone-16α-carbonitrile; SH, small hepatocyte.

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

Received November 16, 2005; accepted July 19, 2006

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 cytochromes P450 (P450s), whether P450 expression was induced by appropriate inducers, and whether P450 activities were measurable. 3-Methylcholanthrene (3-MC), phenobarbital (PB), pregnenolone-16α-carbonitrile (PCN), and ethanol were used as inducers for CYP1A1, 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 3-MC and PCN, respectively. Although Matrigel did not up-regulate the enzymatic activity of CYP1A1, 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, although the activities in both were approximately 30% of that of MHs. Profiles of [14C]-testosterone metabolites were examined in cultured MHs and in cryopreserved SHs by high-performance liquid chromatography. 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 P450s after long-term cryopreservation.

This study was supported by grants from the Science and Technology Incubation Program in Advanced Region, the Japan Science and Technology Agency, the Ministry of Education, Culture, Sports, Science and Technology, Japan (14370393, 17390353) to T.M., and Ministry of Health, Labour and Welfare, Health and Labour Sciences Research Grants, Research on Advanced Medical Technology to T.M.

Materials and Methods

Isolation and Culture of SHs. F344 rats (Sankyo Lab Service, Tokyo, Japan), weighing 170 to 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 described previously (Mitaka et al., 1999). Finally, 6 × 10^3 viable cells/cm² were seeded on a 100-mm dish and cultured in Dulbecco’s modified Eagle’s medium (DMEM; Sigma Chemical Co., St. Louis, MO) supplemented with 20 mM HEPES, 25 mM NaHCO₃, 30 mg/ml l-proline, 10% fetal bovine serum (HyClone, Logan, UT), 10 mM nicotinamide (Katayama Chemical Co., Osaka, Japan), 1 mM ascorbic acid 2-phosphate (Wako Pure Chemical Co., Osaka, Japan), 10 ng/ml epidermal growth factor (Collaborative Research Inc., Lexington, MA), 0.5 mg/ml insulin, 10⁻⁷ M dexamethasone, and antibiotics. After 4 days of culture, 1% dimethyl sulfoxide (DMSO; Aldrich Chemical 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 to 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 × 10³ colonies/60-mm dish) were replated on dishes coated with rat-tail collagen. One day after replating, the medium was replaced with serum-free DMEM supplemented with 1% DMSO. Fourteen days after replating, some dishes were treated with Matrigel (1 mg/dish; Becton Dickinson, Bedford, MA), 0.5 mg/l insulin, 10⁻⁷ M dexamethasone, and antibiotics. After 14 days after plating and immediately replated or cryopreserved at ~80°C for more than 1 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 P450 inducer treatment. Cells at 26 days were harvested from dishes and examined for P450 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.

Immunoblotting for P450 Proteins. 3-Methylcholanthrene (3-MC; Wako Pure Chemical Co.), phenobarbital (PB; Wako Pure Chemical Co.), pregnenolone-16α-carbonitrile (PCN; Sigma Chemical Co.), and ethionine (EtOH; Katayama Chemical Co.) were used as inducers for CYP1A2, 2B, 3A, and 2E, respectively. Eight days after Matrigel treatment, fresh medium containing the inducer (5 μM 3-MC, 2 mM PB, 2 μM PCN, or 100 mM EtOH) was added to enhance the P450 expression, the medium containing each inducer was renewed every day for 3 consecutive days before harvest (Fig. 1A).

For immunoblotting, the samples 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 h at 4°C. The cells were scraped and used for protein extraction. Samples (15 μg/lane) were separated by SDS-polyacrylamide gel electrophoresis. Rabbit anti-CYP1A2, anti-CYP3A2, goat anti-CYP2B1, and anti-CYP2E1 (Daichi Pure Chemical Co., Tokyo, Japan) antibodies were used for immunoblots. The details of the method were described previously (Miyamoto et al., 2005).

Enzyme Activity of P450s. For the measurement of P450 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 h at 37°C. The reaction reagents were collected and centrifuged at 12,000g 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 P450 were fluorometrically measured according to the method of Burke and Mayer (1974) with some modifications (Miyamoto et al., 2005). P450 activities were determined by 7-ethoxyresorufin O-deethylation (CYP1A1), testosterone-6β-hydroxylation (CYP3A), and chlorzoxazone 6-hydroxylation (CYP2E1).

NADPH-Cytochrome P450 Reductase Activity. Activity of NADPH-Cytochrome P450 reductase was measured by the method of Yukioka et al. (1983).

Profiles of ¹⁴C-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% fetal bovine serum. The cells were washed with KH buffer twice at 37°C. After addition of 1.5 ml of KH buffer containing 125 μM ¹⁴C-testosterone (Amersham Biosciences Inc., Piscataway, NJ) to the dish, the cells were incubated for 1 h at 37°C. The reaction reagents were filtered by centrifugation (5000g, 5 min, 4°C) with an UltraFree-CL filter (0.45 μm; Millipore, Billerica, MA), and 100 μl was analyzed. Metabolites were detected using HPLC (LC-10ADvp; Shimadzu, Kyoto, Japan) with a Cosmosil 5C18-AR column (4.6 × 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 to 20 min, 20 to 30% B (linear gradient); 20 to 20.5 min, 30 to 70% B (linear gradient); 20.5 to 24 min, 70% B; 24 to 25 min, 70 to 20% B (linear gradient); 25 to 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 to 50 cells were detached from dishes. The colonies were replated on new dishes or cryopreserved for more than 1 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 reported previously (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 heptocyte nuclear factors 4α and 6, and CCAAT enhancer binding protein α and β, which are known to be restrictedly expressed in highly differentiated hepatocytes (Sugimoto et al., 2002). Expression of P450 proteins 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/piled-up within 1 week. This result was coincident with that of subcultured SHs (Miyamoto et al., 2005). We then examined the expression of P450 proteins by immunoblotting. As shown in Fig. 2, A and B, CYP1A1/2 and CYP3A2 were constitutively expressed and not induced by Matrigel in both subcultured and cryopreserved SHs. Expression of CYP2E1 was detected in subcultured SHs treated with PB but not in cryopreserved SHs. In contrast, CYP2E1 was detected in subcultured SHs treated with Matrigel but constitutively expressed in cryopreserved ones.
When SHs were treated with 3-MC and PCN for 4 days, CYP1A1/2 and CYP3A2, respectively, were induced in the cells with Matrigel. However, this induction was not observed in the cells without Matrigel. As shown in Fig. 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 P450 enzymatic activities. As shown in Fig. 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 3-MC 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. Despite 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 P450, 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 Fig. 2C, the activity was observed in both subcultured and cryopreserved SHs, although measured activity was approximately 0.3-fold that of MHs. The results indicated that the reductase activity might affect P450 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

![Image](image_url)

**Fig. 2.** A. Immunoblots for P450 proteins induced by various P450 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-polyacrylamide gel electrophoresis. Relative expression of each P450 was estimated from immunoblots and is presented in B. C. P450 enzymes and NADPH-cytochrome P450 reductase activity. Activities of P450 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 h at 37°C, and reaction reagent was collected. Resorufin, 6β-hydroxytestosterone, and 6-hydroxychlorzoxazone were detected as metabolites. Activities of P450s (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-Cl (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 units/mg (mM/min/mg protein). “N” indicates no protein. Excluding reductase activity of MHs, all data are means ± S.D. from three different experiments. Asterisks indicate significant induction with the inducer or Matrigel (*, p < 0.05; **, p < 0.01; ***, p < 0.001).
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 Fig. 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 peaks of the heights 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 P450 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 differentiated functions, especially P450 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, can 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.

Acknowledgments. We thank Drs. Yan-Jun Jia and Hideki Oshima for suggestive discussions, and Makiyo Uchida, Chieko Doi, and Minako Kuwano for technical assistance.

References

FIG. 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 h at 37°C. The reaction reagents were analyzed using HPLC. The main metabolites of testosterone catalyzed by P450s are indicated by arrows.

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