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EXPRESSION AND REGULATION OF DRUG METABOLIZING ENZYMES IN AN IMMORTALIZED RAT HEPATOCYTE CELL LINE

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Abstract—A hepatic cell line has been immortalized after simian vacuolating virus 40 infection of adult rat hepatocytes maintained in defined culture conditions. This cell line, designated SVHep B4, expressed nuclear large T antigen, exhibited an extended lifespan (50 subcultures) and had a hepatocyte-like morphology. Expression and regulation of drug metabolizing enzymes were studied in long-term cultures of SVHep B4 cells. Significant activities of phase I and phase II enzymes were detected. γ-Glutamyltransferase, a marker often increased in neoplastic and dedifferentiated hepatocytes, showed a low activity whereas the hepatospecific enzyme tyrosine aminotransferase was expressed at levels similar to those in liver. Responsiveness of drug metabolizing enzymes to inducers was investigated with phenobarbital, dexamethasone and methylcholanthrene. IIB and IA subfamilies of cytochrome P450 were increased, respectively, by phenobarbital (170%) and methylcholanthrene (500%). Glucuronidation of 1-naphthol was increased by phenobarbital (140%) and 3-methylcholanthrene (160%). Phenobarbital, methylcholanthrene and dexamethasone were found to increase significantly γ-glutamyltransferase while tyrosine aminotransferase activity was enhanced by dexamethasone. Stable expression and inducibility of drug metabolizing enzymes in long-term cultures of the SVHep B4 cell line demonstrate that immortalization of adult hepatocytes represents a promising tool for drug biotransformation studies *in vitro*.

Primary cultures of adult hepatocytes represent a useful *in vitro* model for assessment of hepatotoxicity [1], metabolism of xenobiotics [2] and liver gene expression and regulation [3]. However, these studies are possible for only limited periods of time. As a matter of fact, the main alteration which occurs in culture is the loss of hepatospecific functions, such as albumin secretion [4] and drug metabolizing enzyme activities [5]. Cytochrome P450 and associated mixed function oxidases undergo the most pronounced decrease in activity [6]. Culture conditions including hormone-supplemented media [7], extracellular matrices [8] and co-culture systems [9] are known to partially circumvent this dedifferentation. Furthermore, stabilization of differentiation was observed when EDTA was used instead of collagenase for hepatocyte isolation [10, 11]. We demonstrated recently that this stabilization could be extended to a wide range of functions including those of phase I and II drug metabolizing enzymes [12]. Despite these improvements, dedifferentiation is only delayed and hepatocytes are not able to proliferate. Moreover, the difficulties of routine establishment and the occurrence of inter-individual variations, especially in the case of human hepatocytes, emphasize the necessity of an easily available and reproducible model.

Hepatoma cell lines exhibit such characteristics. However, their use is restricted to the expression of specific functions [13], as they often show phenotypic alterations [14, 15]. Thus, hepatocyte immortalization in defined culture conditions aims to establish differentiated and stable hepatic cell lines suitable for pharmaco-toxicological studies. During the past decade, adult and fetal rat hepatocytes have been immortalized successfully mainly with SV40† virus, which possesses a reduced (5.3 kb) and well characterized genome [16]. However, early immortalization experiments with adult hepatocytes yielded dedifferentiated cell lines [17]. More recently, culture conditions have been shown to counteract this dedifferentiation and, thus, influence the immortalization frequency and the final phenotype expressed. As a matter of fact, when hepatocytes were cultured on chemically defined medium during immortalization, the number of proliferating colonies decreased but the cell lines expressed liver-specific functions [18]. So, although differentiation is known to be associated with the quiescent state of the adult hepatocyte [19], it seems possible to obtain proliferating hepatocyte cell lines with a differentiated phenotype. However, the enzymatic system of xenobiotic biotransformation has never been investigated, to our knowledge, in previous SV40-hepatocyte cell lines.

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† Abbreviations: DMEM, Dulbecco's modified Eagle medium; Dex, dexamethasone; EROD, ethoxyresorufin *O*-deethylase; GGT (EC 2.3.2.2), γ-glutamyltransferase; GST (EC 2.5.1.18), glutathion-*S*-epoxide transferase; MC, 3-methylcholanthrene; mEH (EC 3.3.2.2), microsomal epoxide hydrolase; PB, phenobarbital; PROD, pentoxyresorufin *O*-dependentylase; SV40, simian vacuolating virus 40; TAT (EC 2.6.1.5), tyrosine aminotransferase; SDS–PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis; UDPGT (EC 2.4.1.17), uridine diphosphate glucuronosyltransferase; DMSO, dimethyl sulfoxide.

The aim of the present study was to establish differentiated hepatocyte cell lines suitable for drug metabolism studies. The first step was the SV40 immortalization of adult rat hepatocytes in culture conditions allowing prolonged retention of a differentiated phenotype. The second step was the study of expression and regulation of drug metabolizing activities in an established hepatocyte cell line. To our knowledge, the present work represents the first report of drug metabolizing enzyme expression and regulation studies in SV40-immortalized hepatocytes.

MATERIALS AND METHODS

Materials. Adult male Wistar rats weighing 200–300 g (Iffa Credo, Saint Germain L'Arbresle, France) were fed *ad lib.* until time of perfusion. Wild type SV40 virus (LP strain) was a kind gift of Dr Cassingena (Laboratoire de Génétique Cellulaire, Villejuif, France). Collagen, insulin/transferrin/selenite supplement, resorufin and all other chemicals were from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and, where appropriate, were of tissue culture grade. Ethoxy- and pentoxyresorufin (dissolved in DMSO), cytochrome *c* and L-γ-glutamyl-3-carboxy-4-nitranilide were from Boehringer (Mannheim, F.R.G.). [^{14}C]1-Naphthol (58 Ci/mol) was from Amersham International (Amersham, U.K.). Tritiated *cis*-stilbene oxide and *trans*-stilbene oxide were gifts of Prof. Hammock (Departments of Entomology and Environment Toxicology, University of California, U.S.A.). DMEM and antibiotics were from Gibco BRL (Cergy Pontoise, France). Peroxidase labelled antibodies were purchased from Nordic (Tilburg, The Netherlands). Fetal calf serum was provided by Flow Laboratories (Puteaux, France). Culture materials were obtained from Falcon (Becton-Dickinson, Grenoble, France). Surgical instruments, tubing, buffers and glassware used for liver perfusion were sterilized before use.

Hepatocyte isolation. The *in situ* perfusion with EDTA as the dissociating agent was performed as described previously [12]. After Trypan blue exclusion test, hepatocytes were resuspended in complete culture medium for primary culture.

Primary culture and SV40 infection. Hepatocytes (4×10^6) in 3 mL medium were seeded on 60 mm collagen-coated Petri dishes. Cells were suspended in DMEM containing penicillin (100 units/mL), streptomycin (100 μg/mL), fungizone (0.25 μg/mL), fetal calf serum (5% v/v), insulin/transferrin/selenite supplement (bovine pancreas insulin and human transferrin, 5 μg/mL; selenite, 5 ng/mL) and Dex (10^{-7} M). The medium was changed 6 hr after plating to remove unattached cells. Monolayers were washed twice with fresh medium and infected (multiplicity: 100 PFU per cell) with the wild type SV40 strain LP [20]. The medium was renewed 3 hr later and from then on daily.

After 15 days, hepatocyte monolayers were recovered by trypsinization and seeded on 24-well culture plates (collagen coated) at a cell density of 1000 cells/mL.

Initiation and culture of hepatocyte cell lines. When a colony of epithelial cells reached confluence in a well, it was harvested and transferred to a 35 mm dish. At confluence, cells were amplified in 25 and then 75 cm^2 flasks for freezing of early passages (liquid nitrogen storage) and continuous culture.

Two immortalization experiments yielded one and three stably proliferating cell lines. The first cell line, designated SVHep B4, was characterized further in this study and the three other cell lines were amplified and stored in liquid nitrogen for future investigation.

For basal enzyme activity determination, 1.5×10^6 cells were seeded in 60 mm dishes and harvested by scraping after 2 days of culture. Cells were seeded at a lower density (2×10^6 per 100 mm dishes) for induction experiments. The medium was replaced 24 hr after plating by fresh medium containing inducer or vehicle alone (DMSO 0.1% v/v). Phenobarbital (2×10^{-3} M) was dissolved in complete medium, while dexamethasone or 3-methylcholanthrene (5×10^{-6} M) was dissolved in DMSO and added to the medium at a final concentration of 0.1% DMSO. No cytotoxicity was observed for the concentrations of inducer used. Monolayers were harvested by scraping, 72 hr after seeding. Induction experiments were performed at passage 31 (activity evaluation) or at passage 50 (microsome preparation for Western blot analysis). The pellets were washed twice with cold PBS and stored at −80° until analysis. The following determinations were performed on cell homogenates obtained with a Dounce homogenizer, except for TAT which was measured on supernatants of Triton X-100 (0.5% v/v) dissociated cells.

Spectral determination of cytochrome P450. The total amount of cytochrome P450 was measured by second derivative spectroscopy [21].

Enzyme activities determination. The activities of EROD and PROD, which are model substrates for the IA and IIB subfamilies of cytochrome P450, respectively, were determined as described by Burke *et al.* [22]. mEH and GST were evaluated according to Wixtröm and Hammock [23]. NADPH-cytochrome P450 reductase was measured by the method of Strobel and Dignam [24] with cytochrome *c* as the electron acceptor. GGT activity was determined according to Szasz [25] with L-γ-glutamyl-3-carboxy-4-nitranilide as the donor and glycyl-glycin as the acceptor. UDPGT measured with 1-naphthol was evaluated as described by Bock and White [26]. Bilirubin UDPGT activity was followed by the technique of Heirwegh *et al.* [27] and glucuronidation of testosterone according to Rao *et al.* [28]. TAT was measured by the method of Diamondstone [29].

Protein content. Protein concentration was quantified by the method of Bradford [30] with bovine serum albumin as standard.

All activities in SVHep B4 cells represent the means ± SD of assays performed in duplicate on three culture dishes, whereas the activities of freshly isolated hepatocytes are the means ± SD of at least three animals. For induction evaluation, statistical analysis was performed with unpaired two-tailed Student's *t*-test. A difference was considered significant when $P < 0.01$.

Western blotting. Microsomes were prepared from pools of cultures treated with inducers (at least 250×10^6 cells/treatment) at the 50th passage, as

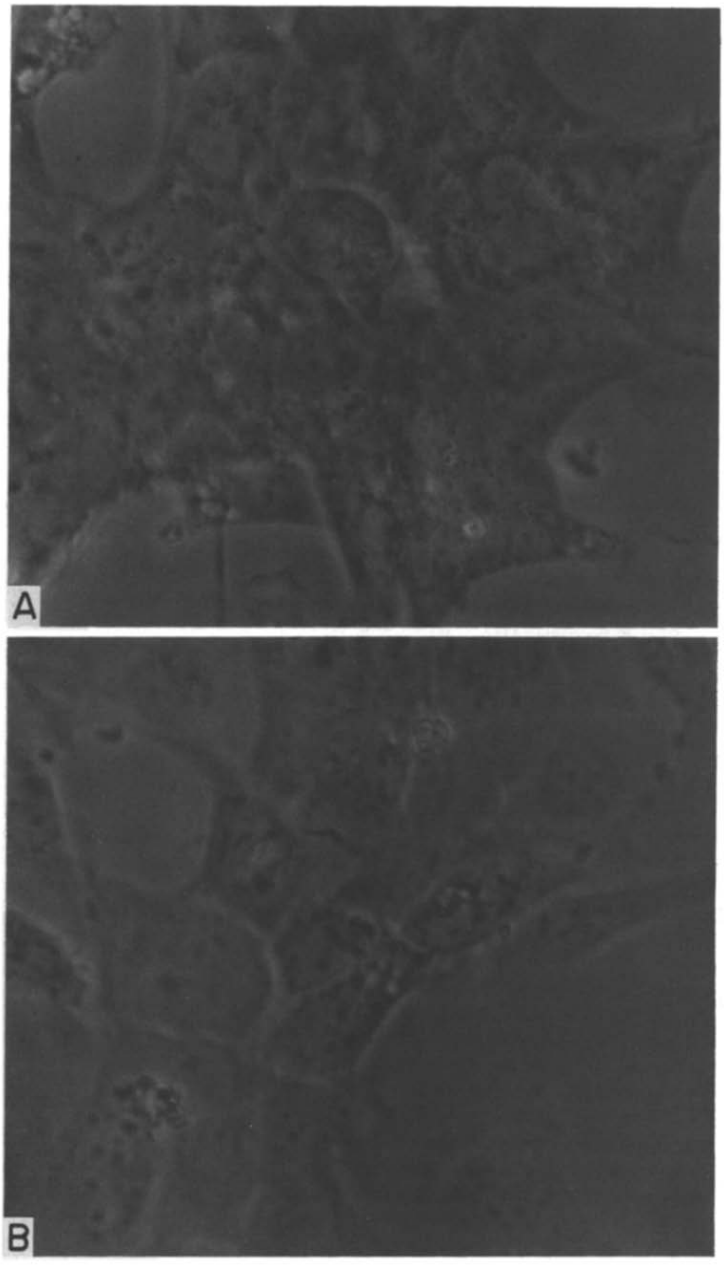

Fig. 1. Morphology of monolayer cultures of SVHep B4 cells. Photomicrographs were performed at passages 5 (A) and 50 (B); 48 hr of culture, ×200.

previously described [31]. Samples (0.03 mg/lane) were submitted to 10% SDS–PAGE according to Laemmli [32]. Blotting on nitrocellulose membranes was performed as described by Gershoni and Palade [33].

Polyclonal antibodies produced in our laboratory against IA and IIB P450 subfamilies [34] were used for immunodetection before incubation with a peroxidase-labelled second antibody.

* Bayad J, Sabolovic N, Bagrel D, Cassingena R and Siest G, Establishment of a differentiated hepatic cell line in defined culture conditions, submitted.

RESULTS

Establishment of hepatic cell lines

Two immortalization experiments yielded one (SVHep B4) and three stably proliferating cell lines, respectively. All immortalized cells showed similar parenchymal morphology and formed monolayers as primary cultures of rat hepatocytes (Fig. 1A). This morphology was maintained for up to 50 subcultures (Fig. 1B). SVHep B4 cells, which were characterized further, exhibited a nuclear expression of viral T-antigen and differentiation markers such as plasma proteins or transaminases, and showed anchorage-dependent growth*. The other immor-

Table 1. Evolution of basal enzyme activities in long-term cultures of SVHep B4 cells

Enzyme activity	Freshly isolated hepatocytes*	SVHep B4† Passage 7	Passage 20	Passage 31
Phase I				
Cytochrome P450‡	115 ± 30	16.8 ± 2.5	19.8 ± 3.1	21.8 ± 2.1
EROD§	21.6 ± 3.4	0.45 ± 0.06	0.49 ± 0.05	0.59 ± 0.04
PROD§	6.9 ± 1.5	1.40 ± 0.10	1.60 ± 0.12	1.43 ± 0.08
NADPH cytochrome P450 reductase‖	14.1 ± 1.1	16.8 ± 2.1	14.4 ± 3.2	16.7 ± 2.5
Phase II				
1-Naphthol UDPGT‖	3.8 ± 0.2	5.8 ± 0.3	5.4 ± 0.3	3.5 ± 0.4
Bilirubin UDPGT§	87.5 ± 5.3	50.1 ± 2.2	42.1 ± 3.9	—
Testosterone UDPGT‖	3.5 ± 0.4	—	—	3.1 ± 0.5
mEH§	600 ± 10	—	—	110 ± 15
GST§	913 ± 76	—	—	850 ± 150
Differentiation markers				
TAT‖	12.6 ± 3.9	8.2 ± 2.8	10.3 ± 1.9	9.1 ± 1.4
GGT‖	1.1 ± 0.2	4.8 ± 0.7	4.9 ± 0.8	4.8 ± 0.9

* Values represent the activities of freshly EDTA-isolated hepatocytes from male Wistar rats (mean ± SD of at least three animals).

† Values are the means ± SD of assays performed on three culture dishes.

Units are: ‡ pmol/mg protein, § pmol/min/mg protein, ‖ nmol/min/mg protein.

—, Not determined.

talized hepatocytes were amplified up to passage 5 and stored in liquid nitrogen for future investigation. The long-term stability of drug metabolizing enzyme expression and inducibility in culture was followed in SVHep B4 cells.

Phenotypic characterization of SVHep B4 cell line

Phase I and II drug metabolizing activities, as well as TAT and GGT activities, were determined at different passages in culture and compared with the activities of freshly isolated hepatocytes. The results were summarized in Table 1.

Although lower than in freshly isolated hepatocytes, phase I drug metabolizing activities were expressed at significant levels, especially in the case of PROD and NADPH cytochrome P450 reductase activities. Among phase II enzymes, 1-naphthol UDPGT was expressed in passages 7, 20 and 31 to a similar extent as in freshly isolated cells. Bilirubin UDPGT activity represented 50% of the hepatocyte activity after both 7 and 20 passages, whereas GST and testosterone UDPGT measured at passage 31 showed activities similar to those of freshly isolated hepatocytes. On the other hand, mEH activity represented only 20% of the activity found in freshly isolated cells.

TAT activity was similar to that in fresh cells and was expressed stably in passages 7, 20 and 31. GGT

Table 2. Effects of inducers on the levels of drug metabolizing enzymes and differentiation markers in SVHep B4 cells

Enzyme activity	Control	Treatment DMSO	PB	Dex	MC
Phase I					
Cytochrome P450*	21.8 ± 2.1	19.7 ± 0.5	33.1 ± 2.2§	33.6 ± 1.1§	22.5 ± 1.6
EROD†	0.59 ± 0.04	0.42 ± 0.04	1.47 ± 0.2§	0.51 ± 0.03	2.54 ± 0.23§
PROD†	1.43 ± 0.08	1.60 ± 0.2	2.40 ± 0.25§	1.01 ± 0.07	1.30 ± 0.15
NADPH cytochrome P450 reductase‡	16.7 ± 2.5	17.9 ± 2.6	30.6 ± 2.8§	32.3 ± 3.8§	20.4 ± 3.9
Phase II					
Naphthol UDPGT‡	3.50 ± 0.40	3.49 ± 0.27	4.62 ± 0.40§	2.95 ± 0.31	5.32 ± 0.35§
Testosterone UDPGT‡	3.10 ± 0.50	2.72 ± 0.44	3.79 ± 0.41	3.38 ± 0.52	2.60 ± 0.22
mEH†	110 ± 15	106 ± 7	202 ± 13§	125 ± 7	103 ± 9
GST†	850 ± 150	780 ± 96	1046 ± 190	983 ± 102	685 ± 106
Differentiation markers					
TAT‡	9.1 ± 1.4	9.4 ± 0.6	10.7 ± 0.8	15.4 ± 1.5§	7.3 ± 0.6
GGT‡	4.8 ± 0.9	4.5 ± 0.4	7.9 ± 0.5§	8.1 ± 0.7§	9.6 ± 0.5§

Control cells (fed with standard medium) are compared with DMSO- (0.1% v/v), PB- (2 mM), Dex- (5 μM) and MC- (5 μM) treated cells as described in Materials and Methods. Values, measured at the 31st passage, represent the means ± SD of three culture dishes.

Units are: * pmol/mg protein, † pmol/min/mg protein, ‡ nmol/min/mg protein.

§ Significantly different from the control cultures ($P < 0.01$).

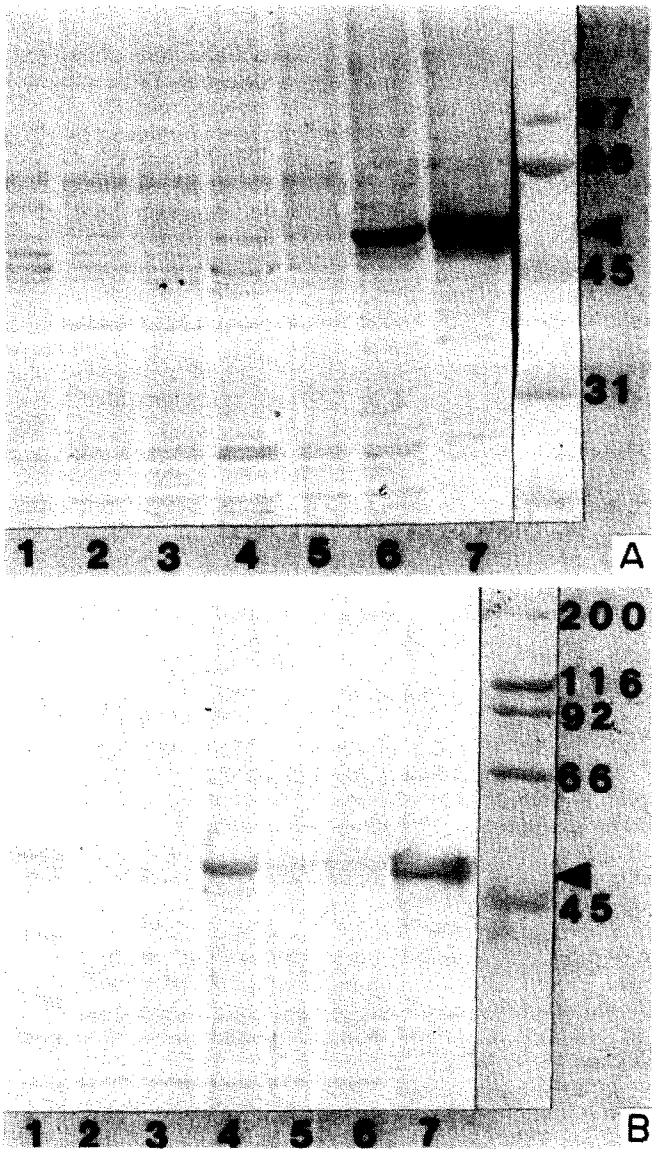

Fig. 2. Induction of IA (A) and IIB (B) subfamilies of cytochrome P450 proteins. For Western blot analysis, solubilized microsomal proteins (30 μg per lane) were separated on 10% SDS–PAGE, blotted on nitrocellulose, hybridized with specific antibodies and revealed with peroxidase-labelled antibodies. Lane 1 contained microsomes from freshly EDTA-isolated hepatocytes. The remaining lanes contained microsomes from untreated SVHep B4 cells (lane 2) or from cells treated with DMSO 0.1% v/v (lane 3), PB (lane 4), Dex (lane 5) or MC (lane 6). The concentrations of inducer are reported in the legend of Table 2. Lane 7 represents liver microsomes from: (A) MC- and (B) PB-treated rats. The arrows indicate the molecular mass of cytochrome P450.

activity was slightly increased when compared to that in freshly isolated cells but it remained constant (5.3 ± 0.7 nmol/min/mg protein) up to the 50th subculture. This stability was also observed for EROD (0.49 ± 0.07 pmol/min/mg protein) and PROD (1.2 ± 0.05 pmol/min/mg protein) activities.

Inducibility of drug metabolizing enzymes

The responsiveness of drug metabolizing enzymes in the SVHep B4 cell line towards various inducers was investigated. At the concentration used, DMSO alone had no apparent effect when enzyme activities were compared to those measured in control cultures. The results are presented in Table 2.

Phase I reactions were sensitive to MC, PB and Dex. The total cytochrome P450 amount was increased significantly both by PB (152%) and Dex (154%), whereas MC had no apparent effect. In the case of IA subfamily, MC and PB specifically induced EROD activity 5- and 2.5-fold, respectively. PROD activity was increased only under PB treatment (170%), whereas NADPH cytochrome P450

reductase activity was increased by PB (184%) and Dex (193%) but not by MC.

Among phase II enzymes, only mEH and 1-naphthol UDPGT were significantly increased by PB (190%) and MC (154%), respectively. A slight but significant increase was also observed for 1-naphthol under PB treatment, while testosterone UDPGT was not enhanced by this inducer. GST activity was not increased significantly either by PB, Dex or MC.

TAT activity, used as a control for Dex induction, and GGT activity were also altered by the inducers tested. TAT activity was increased by Dex only (172%), whereas PB (165%), Dex (167%) and MC (199%) were found to enhance GGT level.

The expression and inducibility of cytochrome P450 IA and IIB subfamilies in SVHep B4 were confirmed at passage 50 by Western blot analysis. The immunostaining corresponding to IA subfamily was enhanced by MC (Fig. 2A, lane 6), whereas PB increased the band of IIB subfamily (Fig. 2B, lane 4). The other inducers and DMSO alone had no apparent effects on the expression of these subfamilies.

DISCUSSION

In our immortalization experiments, a low frequency of stably proliferating colonies was observed, when compared to previous reports using SV40 [17, 20]. This may be attributed to the defined culture conditions that we used to maintain differentiation (isolation method, extracellular matrix, medium composition, early infection). As a matter of fact, the use of chemically defined medium has been shown to decrease transformation frequencies [18]. Thus, it appears that culture conditions allowing differentiation probably decrease rat hepatocyte sensitivity to viral immortalization. However, the mechanism of resistance is not elucidated, although a relationship with the expression of cellular oncogenes is probably involved [19].

Although a slight *in vitro* increase in GGT activity was detected in SVHep B4 cells, the level was low when compared to other hepatic cells in culture [20, 35]. As GGT induction often occurs in fully transformed hepatic cells, it has been proposed as a marker for tumor progression [36]. Thus, on the basis of high hepato-specific markers, low GGT activity and morphological criteria, SVHep B4 cells would seem to exhibit a differentiated phenotype.

This is in good agreement with the results of Woodworth *et al.* [18]. Our study with SVHep B4 cells demonstrated that this observation concerns also drug metabolizing enzyme expression. However, the mixed function oxidase system was expressed at lower levels in SVHep B4 cells than in freshly isolated hepatocytes, while differences were less pronounced in the case of NADPH cytochrome P450 reductase, GST and UDPGTs. Nevertheless, cytochrome P450 expression in SVHep B4 cells was higher than in human [37] or rodent hepatoma cell lines [38].

Induction experiments showed that several drug metabolizing enzymes in SVHep B4 cells were sensitive to inducer treatment. The most important inductions were observed with the IA and IIB cytochrome P450 subfamilies under MC and PB treatment, respectively. Among phase II enzymes, mEH was induced by PB and 1-naphthol UDPGT by both MC and PB; GST was not altered. However, the method used to quantify GST in this study did not distinguish between GST isoforms. Thus, a stable level or a small increase of total GST may be the result of the repression of some isoforms and the induction of others. This phenomenon has been shown for the fetal isoform GST-P_i in rat hepatoma cells [15]. Furthermore, this hypothesis was also illustrated in the case of cytochrome P450 in our induction experiments. As a matter of fact, MC had no apparent effect on total cytochrome P450 content, whereas IA subfamily was increased significantly (EROD activity and Western blot analysis).

In the case of long-term culture, SVHep B4 cells seemed to exhibit a stable phenotype as the expression of GGT, EROD and PROD activities was maintained after 50 subcultures, as well as the morphology. Furthermore, regulation of IA and IIB cytochrome P450 subfamilies was retained after 31 (EROD, PROD) and 50 subcultures (Western blot analysis), which correspond to a time period of about 6 months of continuous culture.

The present study emphasizes the influence of culture conditions on the phenotype expressed by immortalized hepatocytes. Thus, hepatocyte immortalization in defined culture conditions seems to be a promising way of providing long-term *in vitro* models for drug metabolism studies. Furthermore, improvements in immortalization conditions (culture conditions, transfection efficiency or vectors with specific immortalizing DNA sequences) should allow higher and stable expression of hepatic functions by cultured hepatic cell lines, especially those of human origin.

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REFERENCES

1. Guguen-Guillouzo C, Gripon P, Vandenberghe Y, Lamballe F, Ratanasavanh D and Guillouzo A, Hepatotoxicity and molecular aspects of hepatocyte function in primary culture. *Xenobiotica* **18**: 773–783, 1988.
2. Chenery RJ, The utility of hepatocytes in drug metabolism studies. In: *Progress in Drug Metabolism* (Eds. Bridges JW and Chasseaud LF), pp. 217–265. Taylor and Francis, London, 1988.
3. Mathis JM, Houser WM, Bresnick E, Cidlowski JA, Hine R, Prough RA and Simpson ER, Glucocorticoid regulation of the rat cytochrome P-450c (P-450 IA1) gene: receptor binding within intron I. *Arch Biophys Biochem* **269**: 93–105, 1989.
4. Miyazaki M, Handa Y, Oda M, Yabe T, Miyano K and Sato J, Long-term survival of functional hepatocytes from adult rat in the presence of phenobarbital in primary culture. *Exp Cell Res* **159**: 176–190, 1985.

5. Croci T and Williams GM, Activities of several phase I and phase II xenobiotic biotransformation enzymes in cultured hepatocytes from male and female rats. *Biochem Pharmacol* **34**: 3029–3035, 1985.
6. Grant MH, Melvin MAL, Shaw P, Melvin WT and Burke MD, Studies on the maintenance of cytochromes P-450 and b_5, monooxygenases and cytochrome reductase in primary cultures of rat hepatocytes. *FEBS Lett* **190**: 99–103, 1985.
7. Dich J, Vind C and Grunnet N, Long-term culture of hepatocytes: effect of hormones on enzyme activities and metabolic capacity. *Hepatology* **8**: 39–45, 1988.
8. Reid LM, Abreu SL and Montgomery K, Extracellular matrix and hormonal regulation of synthesis and abundance of messenger RNAs in cultured liver cells. In: *The Liver: Biology and Pathobiology* (Eds. Arias IM, Jacoby WB, Popper H, Schachter D and Shafritz DA), pp. 717–737. Raven Press, New York, 1988.
9. Guguen-Guillouzo C, Clément B, Baffet G, Beaumont C, Morel-Chany E, Glaise D and Guillouzo A, Maintenance and reversibility of active albumin secretion by adult rat hepatocytes co-cultured with another liver epithelial cell type. *Exp Cell Res* **143**: 47–54, 1983.
10. Meredith JM, Rat hepatocytes prepared without collagenase: prolonged retention of differentiated characteristics in culture. *Cell Biol Toxicol* **4**: 405–425, 1988.
11. Wang SR, Renaud G, Infante J, Catala D and Infante R, Isolation of rat hepatocytes with EDTA and their metabolic functions in primary culture. *In Vitro Cell Dev Biol* **21**: 526–530, 1985.
12. Bayad J, Sabolovic N, Bagrel D, Magdalou J and Siest G, Influence of the isolation method on the stability of differentiated phehotype in cultured rat hepatocytes. *J Pharmacol Methods* **25**: 85–94, 1991.
13. Darlington GJ, Liver cell lines. *Methods Enzymol* **151**: 19–38, 1987.
14. Everson GT and Polokoff MA, HepG2. A human hepatoblastoma cell line exhibiting defects in bile acid synthesis and conjugation. *J Biol Chem* **261**: 2197–2201, 1986.
15. Tahir MK, Guthenberg C and Mannervik B, Glutathione transferases in rat hepatoma cells. *Biochem J* **257**: 215–220, 1989.
16. Martin RG and Chou JY, Simian virus 40 functions required for the establishment and maintenance of malignant transformation. *J Virol* **15**: 599–612, 1975.
17. Isom HC, Tevethia MJ and Taylor JM, Transformation of isolated rat hepatocytes with simian virus 40. *J Cell Biol* **85**: 651–659, 1980.
18. Woodworth C, Secott T and Isom HC, Transformation of rat hepatocytes by transfection simian virus 40 DNA to yield proliferating differentiated cells. *Cancer Res* **46**: 4018–4026, 1986.
19. Leffert HL, Koch KS, Lad PJ, Shapiro IP, Skelly H and De Hemptinne B, Hepatocyte regeneration, replication, and differentiation. In: *The Liver: Biology and Pathology* (Eds. Arias IM, Jacoby WB, Popper H, Schachter D and Shafritz DA), pp. 833–849. Raven Press, New York, 1988.
20. Lafarge-Frayssinet C, Estrade S, Rosa-Loridon B, Frayssinet C and Cassingena R, Expression of gamma-glutamyltranspeptidase in adult rat liver cells after transformation with SV40 virus. *Cancer Lett* **22**: 31–39, 1984.
21. Ghersi-Egea JF, Walther B, Minn A and Siest G, Quantitative measurement of cerebral cytochrome P-450 by second derivative spectrophotometry. *J Neurosci Methods* **20**: 261–269, 1987.
22. Burke MD, Thompson S, Elcome CR, Halpert J, Haaparanta T and Mayer RT, Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: a series of substrates to distinguish between different induced cytochromes P-450. *Biochem Pharmacol* **34**: 3337–3345, 1985.
23. Wixtröm RN and Hammock BD, Membrane-bound and soluble epoxide hydrolases. In: *Methodological Aspects of Drug Metabolizing Enzymes* (Eds. Zakim D and Vessey DA), pp. 4–93. Wiley, New York, 1985.
24. Strobel HW and Dignam JD, Purification and properties of NADPH cytochrome P-450 reductase. *Methods Enzymol* **52**: 89–98, 1978.
25. Szasz G, A kinetic photometric method for serum gamma-glutamyl transpeptidase. *Clin Chem* **15**: 124–136, 1969.
26. Bock KW and White INH, UDP-Glucuronosyltransferase in perfused rat liver and in microsomes: influence of phenobarbital and 3-methylcholanthrene. *Eur J Biochem* **46**: 451–459, 1974.
27. Heirwegh KPM, Van Der Vijver M and Fevery J, Assay and properties of digitonin-activated bilirubin uridine diphosphate glucuronosyltransferase from rat liver. *Biochem J* **129**: 605–618, 1972.
28. Rao GS, Haneter G, Rao ML and Brever H, An improved assay for steroid glucuronyltransferase in rat liver microsomes. *Anal Biochem* **74**: 35–40, 1976.
29. Diamondstone TI, Assay of tyrosine transaminase activity by conversion of *p*-hydroxyphenylpyruvate to *p*-hydroxybenzaldehyde. *Anal Biochem* **16**: 395–401, 1966.
30. Bradford M, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
31. Turner NA and Pitot HC, Dependence of the induction of cytochrome P-450 by phenobarbital in primary cultures of adult rat hepatocytes on the composition of the culture medium. *Biochem Pharmacol* **38**: 2247–2251, 1989.
32. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **272**: 680–685, 1970.
33. Gershoni GM and Palade GE, Protein blotting: principles and applications. *Anal Biochem* **130**: 416–419, 1983.
34. Souhaili-El Amri H, Fargetton X, Benoit E, Totis M and Batt AM, Inducing effect of rat liver drug-metabolizing enzymes and metabolite pharmacokinetics. *Toxicol Appl Pharmacol* **92**: 141–149, 1988.
35. Levy M, Bagrel D, Sabolovic N, Galteau MM, Wellman M, Rolin S and Siest G, Effects of ethanol on gamma-glutamyltransferase of a rat hepatoma cell line. In: *Liver Cells and Drugs* (Ed. Guillouzo A), pp. 451–458. Libbey Eurotext, London, 1988.
36. Hanigan MH and Pitot HC, Gamma-glutamyltransferase: its role in hepatocarcinogenesis. *Carcinogenesis* **6**: 165–172, 1985.
37. Grant MH, Duthie S, Gray AG and Burke MD, Mixed function oxidase and UDP-glucuronyltransferase activity in the human Hep G2 hepatoma cell line. *Biochem Pharmacol* **37**: 4111–4116, 1988.
38. Frey AB, Rosenfeld MG, Dolan WJ, Adesnik M and Kreibich G, Induction of cytochrome P-450 isozymes in rat hepatoma-derived cell cultures. *J Cell Physiol* **120**: 169–180, 1984.