Biochemical Pharmacology, Vol. 44, No. 11, pp. 2259–2262, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

SHORT COMMUNICATIONS

Modulation of multidrug resistance gene expression in rat hepatocytes maintained under various culture conditions

(*Received* 26 *June* 1992; *accepted* 15 *September* 1992)

Abstract—P-glycoprotein (P-gp), the multidrug resistance gene product, is overexpressed in normal adult rat hepatocytes under standard culture conditions. We have studied the modulation of P-gp expression in this *in vitro* model in the presence of both epidermal growth factor and pyruvate, which favor hepatocyte growth, as well as in the presence of either dimethyl sulfoxide (DMSO) or nicotinamide, which favors maintenance of differentiated functions. P-gp overexpression, estimated by northern blotting and doxorubicin-mediated drug efflux analyses, was similarly observed during culture in both standard and proliferating conditions, while it was delayed, but not inhibited, in the presence of DMSO or nicotinamide. These results suggest that the functional P-gp overexpression occurring in rat hepatocytes when exposed to an unfamiliar environment is at least partly not related to cell proliferation or the degree of cell differentiation *in vitro*.

Multidrug resistance in tumor cell lines is usually related to the overexpression of a plasma membrane glycoprotein termed P-glycoprotein (P-gp*) [1, 2]. P-gp, encoded by *mdr* gene(s), is thought to act as an ATP-dependent efflux transmembrane pump and thus reduces intracellular antitumor drug accumulation in resistant cells [3]. Elevated levels of P-gp have been demonstrated in many human cancers [4] and are correlated with chemotherapeutic outcome and poor clinical prognosis [5]. P-gp is also expressed on the apical membrane of cells facing an excretory compartment in several normal tissues, including the liver, kidney and gastrointestinal tract. This localization suggests that the *mdr* gene product may have a physiological role in transporting cytotoxic compounds or metabolites [6].

Various factors, including hormones and environmental circumstances, have been shown to regulate P-gp expression. The *mdr* gene product is induced in the secretory endometrium of the gravid mouse uterus [7] after heat shock, treatment by heavy metals [8] or chemotherapeutic agents [9], or in response to carcinogens in the liver [10]. P-gp is also markedly overexpressed and associated with increased resistance to antitumor drugs in rat hepatocytes when maintained in primary culture under standard conditions without prior exposure to xenobiotics [11]. Since P-gp expression has been related to the degree of differentiation of human tumors and cell lines [12] and to cell division during liver regeneration after partial hepatectomy [10], we decided to investigate the modulation of P-gp expression in cultured rat hepatocytes by using culture conditions which induce cell growth or favor maintenance of differentiated functions.

Materials and Methods

Cell isolation and culture. Hepatocytes from adult male Sprague–Dawley rats weighing 180–200 g were isolated by the two-step collagenase perfusion method [13]. They were seeded at a density of 10^5 cells per cm^2 on plastic dishes in a medium consisting of 75% minimal essential medium and 25% medium 199, supplemented with 0.2 mg/mL bovine serum albumin, 10 μg/mL bovine insulin and 10% fetal calf serum. Four hours after cell seeding the medium was discarded and replaced by a serum-free medium without or with supplement(s). Some cultures were maintained either in standard conditions as described elsewhere [11] or in the presence of both epidermal growth factor (EGF) (50 ng/mL) and pyruvate (20 mM) which favor hepatocyte growth [14]. Another set of cultures were maintained in the presence of either 2% dimethyl sulfoxide (DMSO) (v/v) or 25 mM nicotinamide; both compounds prevent the loss of adult liver-specific functions during culture [15, 16].

Assay of DNA synthesis. Cultured hepatocytes were incubated in the presence of 1 μCi/mL [^{3}H]thymidine (25 Ci/mmol, Amersham, U.K.) during the 4–24, 24–48, 48–72 and 72–96 hr culture periods. Measurements of DNA synthesis were carried out using scintillation counting of [^{3}H]thymidine incorporated into DNA.

Isolation of RNA and blot analysis. Total RNA was extracted from cultured hepatocytes by the guanidium thiocyanate/cesium chloride method [17]. For northern blotting, 10 μg of total RNAs were subjected to electrophoresis in a denaturing formaldehyde/agarose gel and transferred onto Hybond N sheets (Amersham). Transfer efficiency was verified by staining the gel with ethidium bromide. The sheets were prehybridized and hybridized with ^{32}P-labeled probes. After hybridization, the sheets were washed, dried and autoradiographed at −80°. P-gp mRNAs were detected with pCHP1, a hamster 660 bp cDNA probe [18] (The American Type Culture Collection, Rockville, MD, U.S.A.). Glutathione *S*-transferase 7-7 (GST 7-7), albumin and glyceraldehyde phosphate dehydrogenase (GAPDH) mRNAs were analysed with a rat GST 7-7 (pGSTr7) [19], a rat albumin [20] and a GAPDH [21] specific cDNA probe, respectively.

Measurement of intracellular doxorubicin concentration. The intracellular concentration of doxorubicin was estimated as described previously [22]. Cultured rat hepatocytes were exposed to 25 μM doxorubicin (Roger Bellon laboratories, Neuilly, France) for 2 hr, in the presence or absence of 25 μM verapamil (Sigma Chemical Co., St Louis, MO, U.S.A.). Then, the cells were washed rapidly with phosphate-buffered saline, harvested and ultrasonicated. Proteins were then precipitated with 20% trichloroacetic acid. The acid soluble fraction was used to measure the intracellular concentration of doxorubicin by fluorimetry using excitation and emission wavelengths of 485 and 590 nm, respectively. No toxicity of verapamil or doxorubicin was apparent at the concentrations used over the incubation period. Doxorubicin intracellular accumulation values were analysed by the Student's *t*-test. The criterion of significance of the differences between the means (± standard deviation) was $P < 0.05$.

* Abbreviations: DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; GAPDH, glyceraldehyde phosphate dehydrogenase; GST 7-7, glutathione *S*-transferase 7-7; P-gp, P-glycoprotein.

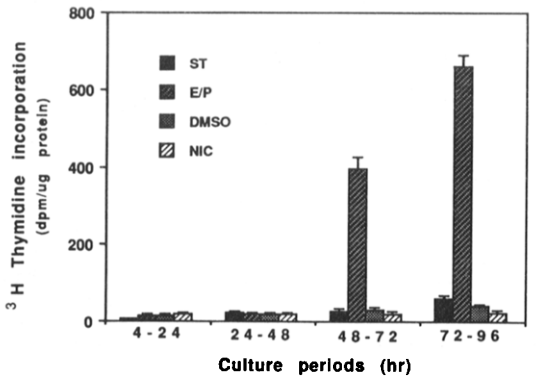

Fig. 1. DNA synthesis in cultured rat hepatocytes. Hepatocytes, maintained under standard conditions (ST) or in the presence of both EGF and pyruvate (E/P), DMSO or nicotinamide (NIC) were incubated with 1 μCi/mL [^{3}H]thymidine. Incorporation of [^{3}H]thymidine into DNA during the 4–24, 24–48, 48–72 and 72–96 hr culture periods was determined using scintillation counting. The values are expressed as dpm/μg protein and are means ± SD of three experiments in triplicate.

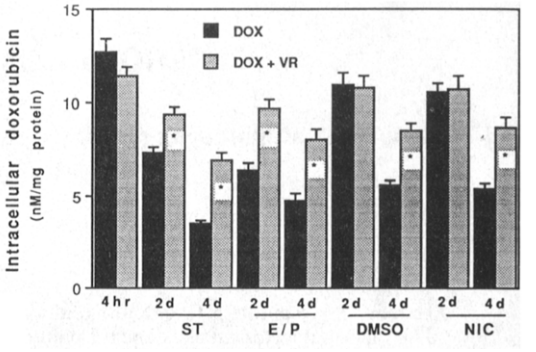

Fig. 3. Intracellular doxorubicin accumulation in cultured rat hepatocytes. Cultured hepatocytes maintained under standard conditions (ST) or in the presence of both EGF and pyruvate (E/P), DMSO or nicotinamide (NIC) were incubated with 25 μM doxorubicin alone (DOX) or 25 μM verapamil (DOX + VR) for 2 hr at 4 hr, 2 days and 4 days after cell seeding. Intracellular doxorubicin concentration was then determined by a fluorimetric method as described in Materials and Methods. The values are expressed as nM/mg protein and are means ± SD of three experiments in triplicate; *$P < 0.05$.

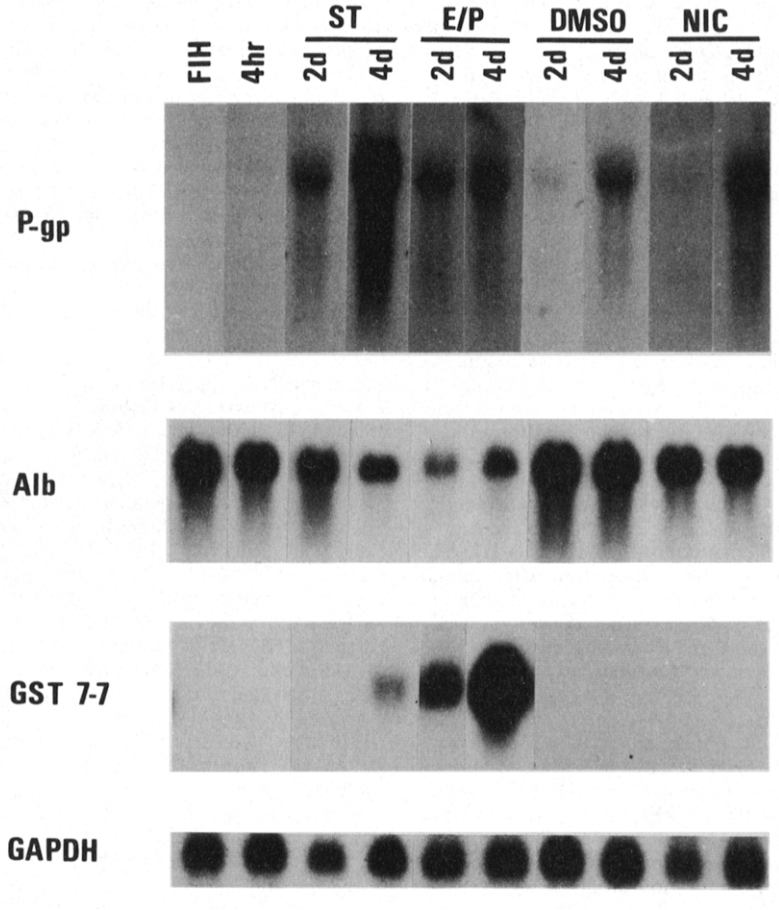

Fig. 2. Expression of P-gp mRNAs in cultured rat hepatocytes. Each well was loaded with 10 μg total RNAs isolated from freshly isolated hepatocytes (FIH), cultured hepatocytes harvested 4 hr, 2 days and 4 days after seeding. Hepatocytes were maintained under standard conditions (ST) or in the presence of both EGF and pyruvate (E/P), DMSO or nicotinamide (NIC). RNAs were transferred onto nitrocellulose sheets after electrophoresis and hybridized with a *mdr* (pCHP1), a GST 7-7 (pGSTr7), a rat albumin (Alb) or a GAPDH specific cDNA probe.

Results

Expression of P-gp in cultured proliferating hepatocytes. Cell growth was measured following [^{3}H]thymidine incorporation performed over the culture period (Fig. 1). Results showed a strong induction of DNA synthesis in the presence of EGF and pyruvate during the 48–72 and 72–96 hr culture periods, thus indicating that hepatocytes proliferated actively in response to supplementation in growth factors. [^{3}H]Thymidine incorporation into DNA was very low whatever the culture period for the hepatocytes, maintained under standard conditions or in the presence of DMSO or nicotinamide (Fig. 1).

As shown in Fig. 2, levels of P-gp mRNAs were strongly augmented in growing hepatocytes compared with freshly isolated or 4 hr cultured hepatocytes. The increase in P-gp expression also occurred as soon as day 2 of culture, as shown in non-proliferating hepatocytes maintained under standard culture conditions.

To determine whether P-gp was functional, cellular accumulation of doxorubicin, an antitumor drug known to be transported by P-gp [1], was measured (Fig. 3). Decreased cellular retention of doxorubicin was found on both days 2 and 4 of culture in hepatocytes maintained under either standard or proliferating conditions. The relationship with P-gp-mediated drug efflux was proved by investigating the effect of verapamil, a compound known to reverse multidrug resistance [23]. At a concentration of 25 μM verapamil increased significantly doxorubicin retention in hepatocytes maintained under either standard or proliferating conditions on both days 2 and 4 of culture (Fig. 3).

Expression of P-gp in cultured differentiated hepatocytes. The cellular differentiation state of cultured hepatocytes maintained in the presence of DMSO or nicotinamide was investigated by estimating the levels of albumin, a specific marker of adult liver parenchymal cells, and of GST 7-7, the GST form expressed in fetal liver and in hepatoma [24]. Albumin mRNA levels remained constant and GST 7-7 mRNAs were undetectable or very low over the culture period (Fig. 2). By contrast, albumin mRNA amounts markedly decreased during primary culture under both standard and proliferating conditions while GST 7-7 mRNAs were strongly induced, particularly in hepatocytes maintained in the presence of EGF and pyruvate (Fig. 2). That equal amounts of RNA were loaded on the gel was demonstrated by hybridization with a GAPDH probe (Fig. 2).

Northern blotting showed that P-gp mRNAs were slightly increased at day 2 of culture and reached high levels only at day 4 of culture in hepatocytes maintained in the presence of DMSO and nicotinamide (Fig. 2). Cellular doxorubicin retention analyses showed that alteration in doxorubicin accumulation and significant action of verapamil were also observed only on day 4 of culture (Fig. 3).

Discussion

Previous studies have demonstrated that culture conditions can greatly affect the phenotype of normal adult rat hepatocytes *in vitro* [25]. Thus, total cytochrome P450 content and the drug metabolic capacity are largely dependent on the culture conditions [25], and the profile of GST is directly related to the composition of the medium [24]. The results reported here show that P-gp expression and P-gp-mediated doxorubicin efflux can also be modulated in liver parenchymal cells by culture conditions. Media which favored maintenance of differentiated functions delayed the increase in functional P-gp occurring during culture.

High levels of functional P-gp expression were found in hepatocytes maintained under both standard and proliferating conditions. In addition, delayed P-gp overexpression in hepatocytes cultured in the presence of DMSO or nicotinamide was observed in the absence of cell growth. Taken together, these data suggest that factors involved in *mdr* gene activation *in vitro* are not directly related to cell proliferation and thus the P-gp overexpression in normal cultured hepatocytes does not reflect that occurring during regeneration after partial hepatectomy [10].

Cellular mechanisms leading to P-gp overexpresion in cultured hepatocytes remain unclear. It is noteworthy that the use of culture media which prevent loss of adult liver-specific functions during culture can delay but not inhibit functional *mdr* gene activation *in vitro*. This favors the idea that the cellular stress resulting from the isolation process and exposure to an unfamiliar environment lead more or less rapidly to a functional P-gp overexpression whatever the culture conditions used and that factors involved in P-gp expression *in vitro* are at least in part not linked to the degree of differentiation of cultured hepatocytes. Comparable levels of P-gp mRNAs were also found in both adult liver and 17 day and 19 day fetal liver (data not shown), suggesting that in the *in vivo* situation P-gp expression in normal hepatocytes is similarly not related to the differentiation status. Activation of *mdr* genes without induction of GST 7-7 in hepatocytes maintained in media which promote maintenance of differentiated functions also favors the idea that these two detoxifying systems are not coordinately regulated in cultured hepatocytes. The same conclusion can be drawn from the data obtained under proliferating conditions. Indeed, GST 7-7 induction was stronger and occurred earlier in the presence of EGF and pyruvate compared to under standard conditions, while P-gp increased concomitantly under both standard and proliferating conditions.

Interestingly, the differentiating agent DMSO which delayed increase in P-gp expression in rat hepatocytes has been demonstrated to enhance *mdr* gene expression in human tumor cell lines [12]. These opposite effects indicate that mechanisms of P-gp regulation are probably quite different in normal versus tumoral cells and/or according to the species and tissue origin.

In summary, P-gp overexpression in cultured rat hepatocytes was similarly observed whether the cells were maintained under standard or proliferating conditions, while it was delayed but not inhibited, in the presence of DMSO or nicotinamide. These results therefore suggest that this P-gp overexpression is not directly related either to the cell growth or to the differentiated state but rather results from exposure of the cells to an unfamiliar environment. Primary rat hepatocyte cultures appear to represent a unique model for analysing P-gp regulation and function in non-tumoral cells.

Acknowledgements—We are grateful to Prof. B. Ketterer for the gift of the GST 7-7 probe, Dr B. Clément for critical reading of the manuscript, and A. Fautrel and M. Rissel for excellent collaboration. This work was supported by INSERM, ARC (Association pour la Recherche sur le Cancer) and the Ligue Nationale Contre le Cancer (Comité d'Ille et Vilaine).

Unité de Recherches Hépatologiques U 49 de l'INSERM Hôpital de Pontchaillou 35033 Rennes France

OLIVIER FARDEL*
PASCAL LOYER
FABRICE MOREL
DAMRONG RATANASAVANH
ANDRÉ GUILLOUZO

* Corresponding author: O. Fardel, INSERM U 49, Hôpital Pontchaillou, 35033 Rennes, Cedex, France. Tel. (33) 99 54 37 37; FAX (33) 99 54 01 37.

REFERENCES

1. Gottesman MM and Pastan I, The multidrug transporter, a double-edged sword. *J Biol Chem* **262**: 12163–12166, 1988.
2. Van Der Bliek AM and Borst P, Multidrug resistance. *Adv Cancer Res* **52**: 165–263, 1989.
3. Cornwell MM, Tsuruo T, Gottesman MM and Pastan I, ATP-binding properties of P-glycoprotein from multidrug-resistant KB cells. *FASEB J* **1**: 51–54, 1987.
4. Goldstein LJ, Galski H, Fojo A, Willingham M, Lai SH, Gazdar A, Pirker R, Green A, Crist W, Brodeur GM, Lieber M, Cossma J, Gottesman MM and Pastan I, Expression of a multidrug resistance gene in human cancer. *J Natl Cancer Inst* **81**: 116–124, 1989.
5. Marie JP, Zittoun R and Sikic BI, Multidrug resistance (mdr1) gene expression in adult acute leukemias: correlations with treatment outcome and *in vitro* drug sensitivity. *Blood* **78**: 586–592, 1991.
6. Thiebaut F, Tsuruo T, Hamada H, Gottesman MM, Pastan I and Willingham M, Cellular localisation of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proc Natl Acad Sci USA* **84**: 7735–7738, 1987.
7. Arceci RJ, Croop JM, Horwitz SB and Housman D, The gene encoding multidrug resistance is induced and expressed at high levels during pregnancy in the secretory epithelium of the uterus. *Proc Natl Acad Sci USA* **85**: 4350–4354, 1988.
8. Chin K-V, Tanaka S, Darlington G, Pastan I and Gottesman MM, Heat shock and arsenite increase expression of the multidrug resistance (MDR1) gene in human renal carcinoma cells. *J Biol Chem* **265**: 221–226, 1990.
9. Chin K-V, Chauhan SS, Pastan I and Gottesman MM, Regulation of mdr RNA levels in response to cytotoxic drugs in rodent cells. *Cell Growth Diff* **1**: 361–365, 1990.
10. Thorgeirsson SS, Huber BE, Sonel S, Fojo A, Pastan I and Gottesman MM, Expression of the multidrug resistance gene in hepatocarcinogenesis and regenerating rat liver. *Science* **236**: 1120–1122, 1987.
11. Fardel O, Ratanasavanh D, Loyer P, Ketterer B and Guillouzo A, Overexpression of the multidrug resistance gene product in adult rat hepatocytes during primary culture. *Eur J Biochem* **205**: 847–852, 1992.
12. Mickley AL, Bates SS, Richert ND, Currier S, Tanaka S, Foss F, Rosen N and Fojo AT, Modulation of the expression of a multidrug resistance gene (mdr-1/P-glycoprotein) by differentiating agents. *J Biol Chem* **264**: 18031–18040, 1988.
13. Guguen C, Guillouzo A, Boisnard M, Le Cam A and Bourel M, Etude ultrastructurale de monocouches d'hépatocytes de rat adulte cultivés en présence d'hémisuccinate d'hydrocortisone. *Biol Gastroenterol* **8**: 223–231, 1975.
14. McGowan JA and Bucher NLR, Pyruvate promotion of DNA synthesis in serum-free primary cultures of adult rat hepatocytes. *In Vitro* **19**: 159–166, 1983.
15. Isom HC, Secott T, Georgoff I, Woodworth C and Mummaw J, Maintenance of differentiated rat hepatocytes in primary culture. *Proc Natl Acad Sci USA* **82**: 3252–3256, 1985.
16. Paine AJ, Hockin LJ and Legg RF, Relationship between the ability of nicotinamide to maintain nicotinamide-adenine dinucleotide in rat liver cell culture and its effect on cytochrome P-450. *Biochem J* **184**: 461–463, 1979.
17. Chirgwin JM, Przybyla EA, MacDonald RJ and Rutter WJ, Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**: 5294–5299, 1979.
18. Riordan JR, Deuchars K, Kartner N, Alar N, Trent J and Ling V, Amplification of P-glycoprotein genes in multidrug resistant mammalian cell lines. *Nature* **316**: 817–819, 1985.
19. Pemble S, Taylor JB and Ketterer B, Tissue distribution of rat glutathione transferase subunit 7, a hepatoma marker. *Biochem J* **240**: 885–889, 1986.
20. Sargent TD, Wu JR, Sala-Trépat JM, Wallace RB, Reyes AA and Bonner J, The rat serum albumin gene: Analysis of cloned sequences. *Proc Natl Acad Sci USA* **76**: 3256–3260, 1979.
21. Fort P, Marty L, Piechaczyk M, Sabrouty SE, Dani C, Jeanteur P and Blanchard J-M, Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family. *Nucleic Acids Res* **13**: 1431–1443, 1985.
22. Schott B, Huet S, Benchekroun MN, Londos-Gagliardi D, Vrignaud P, Montaudan D and Robert J, Pharmacological and molecular characterization of three rodent cell lines in culture selected for resistance to doxorubicin. In: *Anticancer Drugs* (Eds. Tapiero H, Robert J and Lampidis TJ), Vol. 191, pp. 245–252. Les Editions INSERM, Paris & John Libbey Eurotext, London, 1989.
23. Ford JM and Hart WN, Pharmacology of drugs that alter multidrug resistance in cancer. *Pharmacol Rev* **42**: 155–199, 1990.
24. Vandenberghe Y, Morel F, Pemble S, Taylor JB, Rogiers V, Ratanasavanh D, Vercruysse A, Ketterer B and Guillouzo A, Changes in expression of mRNA coding for glutathione *S*-transferase subunits 1-2 and 7 in cultured rat hepatocytes. *Mol Pharmacol* **37**: 372–376, 1990.
25. Guillouzo A, Morel F, Ratanasavanh D, Chesne C and Guguen-Guillouzo C, Long-term culture of functional hepatocytes. *Toxicol In Vitro* **4**: 415–427, 1990.