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Regulation by dexamethasone of P-glycoprotein expression in cultured rat hepatocytes

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We have examined P-glycoprotein (P-gp) expression and function in cultured rat hepatocytes in response to dexamethasone (DEX), which is known to modulate various liver functions. Northern blot analyses revealed high levels of P-gp mRNAs in cultured untreated liver cells in comparison to those found in freshly isolated hepatocytes, while DEX-treated hepatocytes also displayed elevated, although weaker, P-gp levels. Similarly, Western blotting analysis indicated high levels of P-gp in liver cells maintained in the absence of DEX. The use of mdr gene-specific probes allowed us to show that DEX-modulated P-gp induction in cultured hepatocytes involved mostly, if not specifically, mdr1 gene regulation. Doxorubicin P-gp-mediated efflux analyses revealed lower intracellular doxorubicin accumulation in DEX-untreated liver cells than in DEX-treated cells, thus indicating that over-expressed P-gp was functional. These data clearly show that DEX treatment strongly modulates P-gp expression in primary rat hepatocyte cultures through a specific effect on the mdr1 gene.

P-Glycoprotein; mdr gene; Rat hepatocyte; Dexamethasone; Doxorubicin; Primary culture

1. INTRODUCTION

The multidrug resistance (MDR) phenotype is usually linked to over-expression of a plasma transmembrane phosphoglycoprotein termed P-glycoprotein (P-gp) [1–3]. P-gp is thought to act as an ATP-dependent efflux pump and thus confers resistance to various structurally and functionally unrelated cytotoxic drugs. P-gp is encoded by mdr genes which constitute a small gene family comprising two members in humans (MDR1 and MDR2) and three members in rodents (mdr1, mdr2 and mdr3) [4]. Only MDR1 in humans and mdr1 and mdr3 (also known as mdr1b and mdr1a, respectively) in rodents have been demonstrated to be involved in drug resistance by transfection experiments [5].

P-gp is abundant in many drug-resistant human tumors [6,7] and, in addition, is expressed in several normal tissues, including the small intestine, kidney, and liver [8]. P-gp physiological function and regulation remain unclear. In normal hepatocytes, P-gp expression appears to be induced after protein synthesis inhibition, thus suggesting a negative regulation by a labile protein factor [9,10]; it is also increased after partial hepatectomy or xenobiotic treatment [11,12]. Similarly, it is strongly augmented in normal kidney cell lines after acute exposure to cytotoxic drugs [13]. Several studies are in favor of a link with steroids. P-gp localization in the adrenal cortex, in the trophoblast of the human placenta, and in the gravid uterus, suggests that steroid hormones could be endogenous substrates. Moreover, P-gp expression is modulated by estrogen and progesterone in the secretory epithelium of the mouse uterus [14] and in rat pituitary cells [15]. Since various liver functions have been demonstrated to be strongly modulated both in vivo and in vitro by corticosteroid hormones [16], and since primary cultures of rat hepatocytes represent a suitable model to analyze P-gp regulation in non-tumoral cells [17], we decided to investigate P-gp regulation in response to dexamethasone (DEX) treatment in this in vitro model.

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Abbreviations. DEX, dexamethasone; GAPDH, glyceraldehyde phosphate dehydrogenase; GST7-7: glutathione-*S*-transferase 7-7; MDR, multidrug resistance; P-gp, P-glycoprotein.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Doxorubicin hydrochloride was purchased from Roger Bellon laboratories (Neuilly, France). Verapamil and DEX were obtained from Sigma Chemical Co. (St. Louis, MO).

2.2. *Cell isolation and culture*

Hepatocytes from adult male Sprague–Dawley rats weighing 180–200 g were isolated by perfusion of the liver with a collagenase solution as previously described [18]. They were seeded at a density of 10^5 cells/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 mg/ml bovine insulin and 10% fetal calf serum. 4 h after cell seeding the medium was discarded and cultured hepatocytes were then maintained in serum-free medium in the absence or presence of various concentrations of DEX.

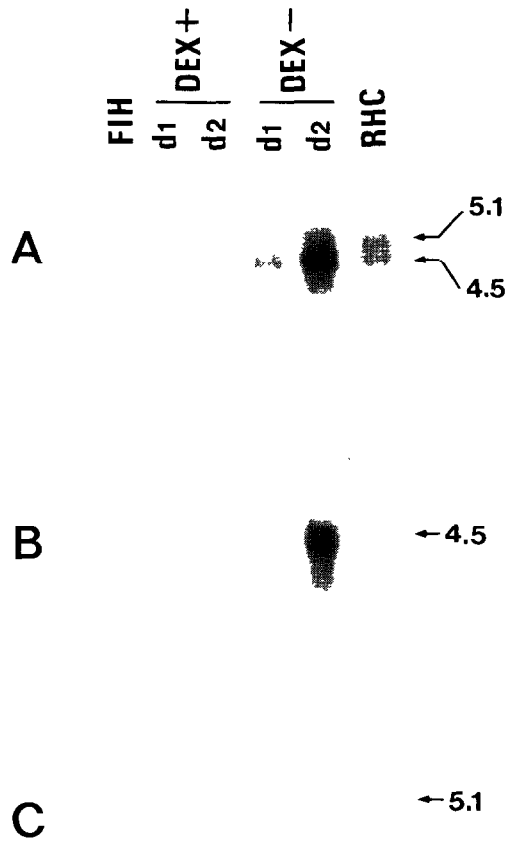

Fig. 1. Effect of dexamethasone (DEX) treatment on P-glycoprotein (P-gp) mRNA levels in cultured rat hepatocytes. Each well contains 10 μg total RNAs prepared from freshly isolated hepatocytes (FIH), hepatocytes cultured with or without DEX (10^{-7} M) and harvested 1 day (d1) or 2 days (d2) after cell seeding, or from multidrug-resistant rat hepatoma cells (RHC). RNAs were transferred to Hybond-N sheets after electrophoresis and hybridized with pCHP1 probe (A), which recognizes highly conserved regions of mdr genes, and with mdr1 (B) and mdr3 (C) gene-specific probes. Transcript sizes (in kb) were estimated relative to the migration of 18 S and 28 S rRNA.

2.3. *Isolation of RNA and blot analysis*

Total RNA was extracted from cultured hepatocytes by the guanidium thiocyanate/cesium chloride method of Chirgwin et al. [19] as modified by Raymondjean et al. [20]. For Northern blotting, 10 μg of total RNAs were subjected to electrophoresis in a denaturing formaldehyde/agarose gel and transferred onto Hybond-N sheets. RNA amount in each lane and transfer efficiency were verified by staining the gel with ethidium bromide. The sheets were prehybridized and hybridized with ^{32}P-labeled probes. P-gp mRNAs were detected with a hamster pCHP1 probe [21] obtained from the American Type Culture Collection (Rockville, MD) and mouse mdr1 BBpG4, mdr2 HIpG3 and mdr3 H3pG3 probes (a generous gift from Dr. P Gros, McGill University, Montreal, Canada). The pCHP1 sequence has been shown to recognize highly conserved regions of mdr genes [22], while BBpG4, HIpG3 and H3pG3 are mdr1-, mdr2- and mdr3-specific probes, respectively [23]. Glutathione-*S*-transferase 7-7 (GST7-7), albumin and glyceraldehyde phosphate dehydrogenase (GAPDH) mRNAs were analysed with rat GST7-7 pGSTr7 [24], albumin cDNA [25] and GAPDH cDNA [26] probes, respectively. After hybridization, the sheets were washed, dried and autoradiographed at *80°C

Total RNAs extracted from rat hepatoma cells isolated in our laboratory and displaying a MDR phenotype associated with over-expression of mdr1 and mdr3 genes (Fardel et al., unpublished results) were used as positive controls for P-gp expression.

2.4. *Preparation of membranes and immunoblotting*

Crude membranes were prepared from cultured hepatocytes by differential centrifugation as described by Germann et al. [27]. Membrane proteins were separated on a SDS-7% polyacrylamide gel and electrophoretically transferred to nitrocellulose sheets. Nitrocellulose sheets were blocked for 2 h with Tris-buffered saline containing 3% bovine serum albumin and sequentially incubated with C219 monoclonal antibody (Centocor Inc., Malvern, PA) raised against P-gp [28] and [^{125}I]protein A (Amersham, Bucks, UK). After washing, blots were dried and autoradiographed at −80°C. A control blot was performed using the same protocol with non-immune myeloma cell ascites as the primary antibody.

2.5. *Evaluation of intracellular doxorubicin concentration*

The intracellular concentration of doxorubicin was estimated as described by Schott et al. [29] with slight modifications. Cultured hepatocytes were exposed to doxorubicin (10 μg/ml) for 2 h and then washed with phosphate buffer, harvested and ultrasonicated. These steps were performed quickly in order to avoid any drug efflux. Proteins were then precipitated with 20% trichloroacetic acid. The acid-soluble part was used to evaluate the intracellular concentration of doxorubicin by fluorimetry, using excitation and emission wavelengths of 485 nm and 590 nm, respectively. Preliminary controls showed no toxicity of doxorubicin at the concentration used over the incubation period. The results of doxorubicin accumulation studies were analysed by Student's *t*-test. The criterion of significance of the differences between the means (±S.D.) was $P < 0.05$.

3. RESULTS

Hepatocytes were cultured for 2 days after cell seeding in the absence or presence of DEX (10^{-7} M). Northern blotting analysis and hybridization with pCHP1

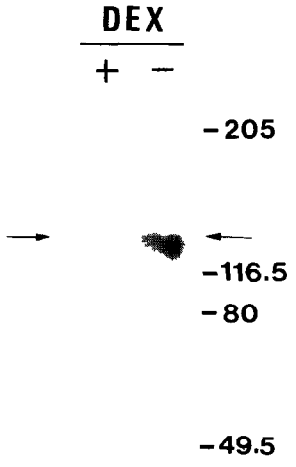

Fig. 2. Western blot analysis of membrane proteins obtained from cultured hepatocytes maintained in the presence or absence of dexamethasone (DEX). Crude membrane fractions were prepared from hepatocytes cultured for 2 days with or without DEX (10^{-7} M). 100 μg membrane proteins were separated on SDS-PAGE and transferred onto a nitrocellulose sheet. After incubation with C219 antibody, the blot was developed as described in section 2. Arrows indicate the position of P-gp. The position of molecular size standards (in kDa) is indicated on the right.

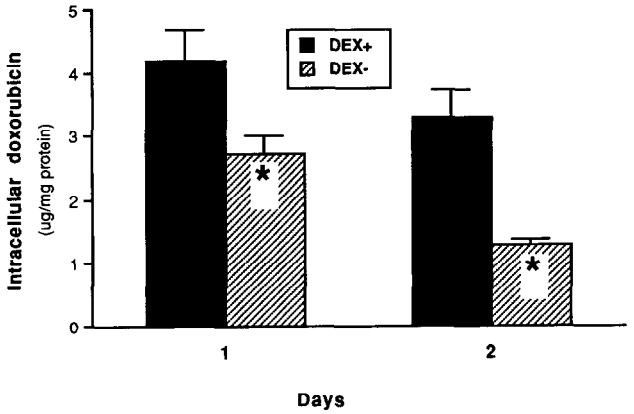

Fig. 3. Intracellular doxorubicin accumulation in hepatocytes cultured with or without dexamethasone (DEX). Rat hepatocytes maintained with or without DEX (10^{-7} M) were incubated with 10 μg/ml doxorubicin for 2 h after 1 and 2 days of culture. The intracellular doxorubicin concentration was then determined using the fluorimetric method, as described in section 2. The values are the mean ± S.D of three experiments in quintuplicate; *$P < 0.05$.

probe revealed high levels of 4.5 kb P-gp mRNA transcripts in hepatocytes maintained without steroid at day 1 and especially at day 2 of culture compared to those found in freshly isolated hepatocytes (Fig. 1). P-gp mRNA levels were also increased in DEX-treated hepatocytes, but to a much lower extent. Hybridizations with mdr1- and mdr3-specific probes revealed enhanced levels of 4.5 kb mdr1 mRNA transcripts in cultured hepatocytes, particularly in the absence of DEX treatment, while 5.1 kb mdr3 mRNAs transcripts were not detected (Fig. 1). Drug-resistant rat hepatoma cells used as positive controls of P-gp expression displayed both increased 4.5 kb mdr1 and 5.1 kb mdr3 mRNA levels, as demonstrated by hybridization with pCHP1 and mdr- specific probes. No signal was detected in either cultured rat hepatocytes or hepatoma cells using the mdr2-specific probe (data non shown).

Crude membranes prepared from cultured rat hepatocytes maintained in the absence or presence of DEX (10^{-7} M) were used to investigate P-gp expression by Western blotting analysis. A C219-reactive band of 140 kDa corresponding to P-gp was strongly expressed in untreated liver cells cultured for 2 days without DEX but much weaker in DEX-treated hepatocytes (Fig. 2).

In order to determine if huge increased P-gp levels in DEX-untreated cultures were associated with functional increased P-gp activity, the cellular accumulation of doxorubicin, an anthracycline antitumor drug known to be transported by P-gp [1], was measured. As shown in Fig. 3, lower intracellular retentions of the anticancer compound were found in DEX-untreated hepatocytes compared to DEX-treated cells at both day 1 and day 2 of culture. Addition of verapamil (25 μM), a drug known to reverse MDR by inhibiting P-gp function [1], led to a significant increase in cellular doxorubicin accumulation (Fig. 4). Coincubation of doxorubicin with various concentrations of DEX for 2 h did not affect cellular anthracycline levels in 2-day-old DEX-untreated hepatocytes (Fig. 4).

The effects of various DEX concentrations on P-gp expression in cultured hepatocytes were further characterized (Fig. 5). Hybridization with pCHP1 probe revealed increased 4.5 kb mRNAs transcripts in rat liver cells maintained in the presence of 10^{-8} M and lower concentrations of DEX for 2 days after cell seeding. Similarly, expression of the fetal liver marker GST7-7 [30] was enhanced while expression of albumin, a specific marker of adult liver parenchymal cells was concomitantly decreased. By contrast GAPDH mRNAs were not affected by DEX treatment whatever the hormone concentration used (Fig. 5).

4. DISCUSSION

The results reported here show that DEX markedly regulates P-gp expression in cultured normal liver cells. Northern blot analysis revealed an early and conspicious increase of P-gp mRNA levels in cultured hepatocytes maintained in the absence of DEX, while DEX-treated cells displayed a weaker P-gp induction at the studied culture times similar to that previously described [17]. Similarly, Western blot analysis indicates high levels of P-gp in liver cells maintained in the absence of DEX.

Two mdr genes, mdr1 and mdr3, have been demonstrated to be involved in drug resistance [5]. The use of mdr gene-specific probes allowed us to detect in liver cell cultures increased levels of mdr1 mRNA transcripts of 4.5 kb, as previously reported for rat mdr1 transcripts [9]. By contrast, Northern blot hybridizations with mdr2- and mdr3-specific probes suggest that both

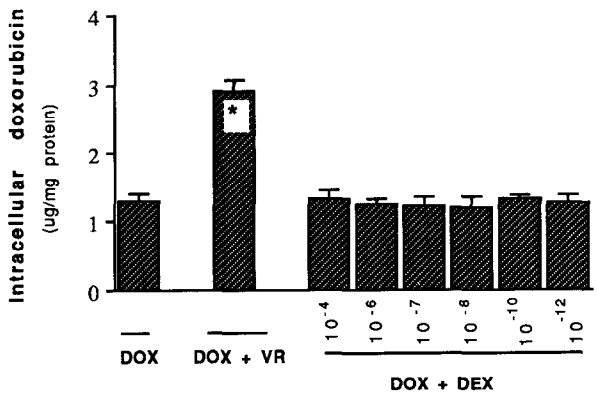

Fig. 4. Effect of verapamil (VR) and dexamethasone (DEX) on doxorubicin retention in hepatocytes cultured without DEX. Rat hepatocytes, maintained in culture without DEX for 2 days after cell seeding, were incubated with 10 μg/ml doxorubicin for 2 h alone or with verapamil (25 μM) or various concentration of DEX (10^{-4}–10^{-12} M). The intracellular doxorubicin concentration was then determined using the fluorometric method, as described in section 2. The values are the mean ± S.D. of three experiments in quintuplicate; *$P < 0.05$.

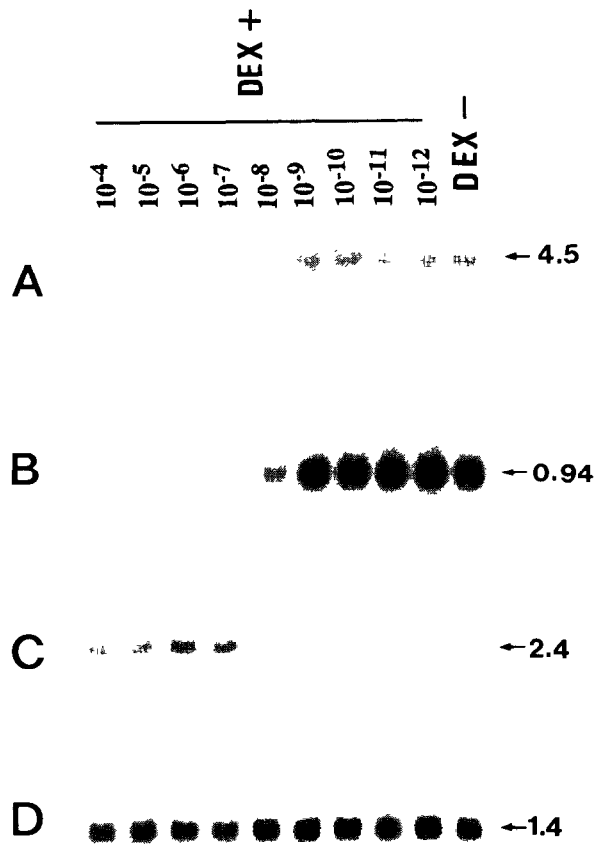

Fig. 5. Expression of P-glycoprotein (P-gp), glutathione-*S*-transferase 7-7 (GST7-7), albumin (Alb) and glyceraldehyde phosphate dehydrogenase (GAPDH) mRNAs in cultured rat hepatocytes exposed to various concentrations of dexamethasone (DEX). Each well contains 10 μg total RNAs prepared from freshly isolated hepatocytes (FIH) or from hepatocytes cultured for 2 days after cell seeding with or without various concentrations of DEX (10^{-4}–10^{-12} M). RNAs were transferred to Hybond-N sheets after electrophoresis and hybridized with a P-gp (pCHP1) (A), a GST7-7 (B), an albumin (C) and a GAPDH (D) probe, respectively. After hybridization, the sheets were washed, dried and autoradiographed at −80°C. Transcript sizes (in kb) were estimated relatively to the migration of 18 S and 28 S rRNA.

mdr2 gene, which is not involved in cytotoxic drug transport, and mdr3 gene were not strongly induced. Consequently it appears that DEX-modulated P-gp induction in cultured hepatocytes involved mostly, if not specifically, mdr1 gene. Increased mdr1 gene expression also occurred in normal liver cells after exposure to 3-methylcholanthrene or 2-acetyl aminofluorene [31]. Taken together, these data strongly suggest that the mdr1 gene could play a critical role in P-gp regulation in normal rat hepatocytes. This conclusion is moreover supported by the recent report demonstrating mdr1 gene induction in normal rat hepatocytes after protein synthesis inhibition through a transcriptional mechanism [9]. We have also observed a strong induction of 4.5 mdr1 mRNA transcripts in response to cycloheximide exposure in liver cells maintained in the presence or absence of DEX (data non shown), thus suggesting that DEX treatment does not involve major change in P-gp regulation in response to protein synthesis inhibition.

The strong induction of P-gp in DEX-untreated hepatocytes paralleled a marked decrease in doxorubicin intracellular retention, thereby indicating that induced P-gp was functional. This low level of anthracycline accumulation in DEX-untreated cultures was strongly enhanced by the addition of the known P-gp modulator agent, verapamil. By contrast, co-incubation of doxorubicin and DEX did not affect the cellular drug level in 2-day-old hepatocyte cultures previously maintained in the absence of DEX, suggesting thus that DEX does not interact with P-gp function. These results also favor the idea that DEX is not a substrate for P-gp in cultured rat hepatocytes. DEX has already been found to be without effect on vinblastine accumulation and does not inhibit [^{3}H]vinblastine binding of membrane vesicles prepared from murine multidrug-resistant cells, in contrast to progesterone [32]. However, similar studies performed in human MDR1-transfected cells have recently shown that DEX, but not progesterone, is a substrate for human P-gp [33]. These conflicting results on DEX transport by P-gp could reflect, in fact, differences in substrate specificity between human and rodent P-gp.

Analysis of the effects of various DEX concentrations demonstrated that maintenance of hepatocyte cultures without DEX or with a low DEX concentration, which resulted in a strong induction of P-gp, also increased expression of the fetal liver marker, GST7-7, and decreased concomitantly the level of albumin, an adult liver-specific function. Taken all together, these data suggest that modulation of P-gp levels in response to DEX treatment could rather be a part of an early and marked alteration of differentiation status in untreated cultures and cultures exposed to a low DEX concentration than a specific action of DEX on mdr 1 gene. A relationship between the differentiation status and P-gp expression in liver cells is also suggested by high levels of mdr mRNAs found in pre-neoplastic and neoplastic liver [11] and by a recent report demonstrating that hepatocytes cultured on a reconstituted basement membrane, termed matrigel, displayed high liver-specific functions and reduced P-gp levels, respectively [34]. However, it is noteworthy that an induction of P-gp, although weak, occurs in DEX-treated cultured hepatocytes. Addition of compounds such as dimethyl sulfoxide and nicotinamide, which favor maintenance of differentiated functions, did not completely abolish the P-gp increase in hepatocyte cultures [17]. This could thus indicate that other factor(s) not directly related to differentiation status, but which could be linked to a cellular stress response resulting from cell isolation and exposure to an unfamiliar environment, is also involved in P-gp induction occurring in cultured rat hepatocytes.

In summary, Northern and Western blot analyses and P-gp-mediated drug efflux studies have demonstrated that DEX modulates P-gp induction in cultured rat hepatocytes. This effect of DEX on P-gp involves regulation of the mdr1 gene; it could be a part of liver cell differentiation status modulation by DEX. Further studies are needed to clarify if DEX treatment could also regulate P-gp expression in drug-resistant tumoral cells, that could then lead to a new and interesting use of steroid hormones for overcoming MDR in cancers.

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