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MODULATION OF P-GLYCOPROTEIN AND *mdr1b* mRNA EXPRESSION BY GROWTH FACTORS IN PRIMARY RAT HEPATOCYTE CULTURE

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Summary: P-glycoproteins encoded by members of the *mdr* gene family function as membrane-situated transport proteins, isoforms of which are involved in conferring a form of multidrug resistance by participating in secretion of various xenobiotics. In primary rat hepatocytes maintained in serum-free culture, accumulation of immunodetectable P-glycoprotein and *mdr1b* mRNA occurred in a time-dependent manner and was accompanied by a substantial decrease in retention of the mdr1 substrate rhodamine 123. However, incubation of cells with epidermal growth factor (EGF) or with insulin-like growth factor I (IGF-I) markedly enhanced time-dependent accumulation of P-glycoprotein and *mdr1b* mRNA. Furthermore, EGF-treated cells exhibited decreased intracellular rhodamine 123 retention, an effect partially inhibited by the chemosensitizer verapamil. These data suggest that an increase in (a) functional transporter(s) eliciting transport of mdr1 substrates occurs under EGF.

The *mdr* gene family comprises three members in rodents (*mdr*1a, *mdr*1b and *mdr*2 in mouse and rat species [1, 2]), all of which encode glycosylated transporter proteins located in the plasma membrane. Apart from acting as transporters of endogenous substrates such as steroid hormones [3] proteins encoded by *mdr1* genes have been shown to mediate extrusion of various hydrophobic drugs, e. g. several groups of naturally occurring cytotoxic compounds [4]; therefore, an increase in mdr1 transport activity can contribute towards development of drug resistance. While the mdr2 isoform has been shown not to mediate transport of anticancer drugs, a physiological role of this isoform in the liver may be to elicit phospholipid transport [5].

Rodent as well as human livers exhibit high P-glycoprotein expression which is concentrated on the canalicular hepatocyte membrane [6, 7]. Although all three mdr isoforms are expressed simultaneously in normal adult rat liver, the mdr2 form is the most abundant [8]. Overexpression of mdr1 isoforms has been observed in rodent liver during cholestasis [9], regeneration following partial hepatectomy [10] and experimentally induced hepatocarcinogenesis [11, 12]. Furthermore, the human homolog MDR1 is overexpressed in hepatocellular carcinoma [13]. Although elevation of *mdr1* gene expression has been shown to occur in response to xenobiotics in rat li-

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Abbreviations: EGF, epidermal growth factor; IGF-I, insulin-like growth factor I; P-gp, P-glycoprotein; PVDF, polyvinylidene difluoride; PMSF, phenylmethylsulfonyl fluoride.

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ver and in hepatocyte culture [14] little is known about modulation by endogenous regulators. Corticosteroid hormones and extracellular matrix proteins may constitute endogenous modulators, as dexamethasone [15] or laminin and type IV collagen [16] inhibit basal *mdr1* expression in rat hepatocyte culture.

EGF-receptor-mediated stimulation of cell proliferation is thought to contribute towards liver regeneration [17] and to play a role in cell growth during hepatocarcinogenesis [18]. In the present communication, we report that EGF acts as a modulator of *mdr1* expression *in vitro*, demonstrating that changes in *mdr* gene expression during different states of differentiation or development may be linked to cellular response to hepatotrophic growth factors.

MATERIALS AND METHODS

Matrix proteins and growth factors. Collagen type I, murine EGF and recombinant human IGF-I were obtained from Boehringer (Mannheim, FRG), bovine insulin and hydrocortisone hemisuccinate from Sigma (Munich, FRG).

Cell culture. Hepatocytes were isolated from adult male Wistar rats (230-250 g b.w.) by collagenase perfusion [19]. Cells suspended in MX-82 medium [20] containing 10% fetal calf serum were seeded on plastic dishes (Nunc, Kamstrup, Denmark) coated with collagen type I (4.1 $\mu g/cm^2$) at a density of 8.6 x 10^4 cells/cm^2 and incubated in humidified atmosphere of 10% CO_2/90% air. Following an initial attachment period of 2 hours, medium was exchanged to serum-free MX-83 medium [20] supplemented with 1 µM insulin and 20 µM hydrocortisone. Growth factors were added to the medium at final concentrations of 0.16-16 nM (EGF) or 0.13-130 nM (IGF-I). Media changes were performed daily. After culture periods of 5 hours to 5 days cells were washed once with PBS, pH 7.4, frozen in liquid nitrogen and stored at -80°C.

Isolation of subcellular membrane fractions. Plasma membrane fractions of approximately 10^7 cells/sample were isolated by differential centrifugation by a modification of the procedure described previously [21] in that buffers used for homogenization and centrifugation contained 1 mM PMSF. The pellet of the resulting plasma membrane fraction was resuspended in 40-400 µl of 10 mM Tris-HCl/1 mM EDTA, pH 7.4, to approximately 1-4 mg protein/ml, homogenized, frozen in liquid nitrogen and stored at -80°C.

Western blot analysis. Cell membrane fractions containing 10 µg protein as determined according to [22] were subjected to SDS-PAGE through 7.5% gels [23] and to subsequent transfer to PVDF membranes (Millipore, Eschborn, FRG) by semi-dry blotting [24], using a modification of the anode buffer (75 mM Tris-HCl/20% methanol, pH 7.4). Immunodetection of P-glycoprotein was performed utilizing primary polyclonal antibody PC03 (Oncogene Science, distributed by Dianova, Hamburg, FRG) raised against a conserved sequence in the C-terminal cytoplasmic P-glycoprotein region (SALDTESEKVVQEALDKAREG) and secondary peroxidase-conjugated antibody (Sigma, Munich, FRG). Visualization of P-glycoprotein bands was achieved by enhanced chemiluminescence employing reagents (peroxidase substrate luminol and Hyperfilm ECL) supplied by Amersham (Braunschweig, FRG).

Northern blot analysis. Total RNA was isolated by guanidinium thiocyanate phenol extraction [25] and separated by electrophoresis through formaldehyde/agarose gels (20 µg RNA/lane). Staining of gels with ethidium bromide was performed to control equal RNA loading of lanes. Subsequently, RNA was transferred to Hybond N nylon membrane (Amersham) by vacuum blotting and hybridized to a rat *mdr1b*-specific antisense oligonucleotide probe end-labeled by T4-polynucleotide kinase (Promega, distributed by Serva, Heidelberg, FRG) using [γ-^{32}P]ATP (DuPont/NEN, Bad Homburg, FRG) [26]. The probe (5'-CTC-AGA-GGC-ACC-AGT-GTC-3') corresponded to *mdr1b*-specific cDNA nucleotides 1936-1953 [2].

Determination of intracellular rhodamine 123 accumulation. Cells grown on 28 cm^2 dishes were incubated with MX-82 medium containing 6.5 µM rhodamine 123 in the presence or absence of 8 µM verapamil for 2 hours at 37°C. Subsequently, adherent cells were washed five times in 4°C cold PBS and intracellular rhodamine was extracted by incubation with n-butanol (2.5 ml/dish) for 10 min at room temperature. Rhodamine 123 concentrations were determined in extracts by fluorometry according to [27] and corrected on the basis of protein content.

RESULTS AND DISCUSSION

Basal P-glycoprotein expression was examined in rat hepatocytes cultivated on type I collagen matrix in the presence of serum-free MX-83 medium supplemented with insulin and hydrocortisone. P-glycoprotein expression increased markedly in a time-dependent manner, reaching peak levels on the third to fourth day of culture (Fig. 1A). Two P-glycoprotein bands were detected in western blot analysis (Fig. 1A), exhibiting mobilities corresponding to apparent molecular weights of approximately 200 kDa and 155 kDa, respectively. These protein species may represent different isoforms and/or posttranslational modifications (e. g. differing states of glycosylation [6]) since the primary antibody employed for immunodetection was directed against a conserved sequence common to all fully sequenced rat P-glycoprotein isoforms (mdr1b and mdr2, [2, 28]).

Mdr1b mRNA, migrating at 4.5 kb, was detected using a novel oligonucleotide probe directed against a gene-specific sequence in the "linker domain" encoding region which exhibits high variability amoung members of the *mdr* gene family [1]. *Mdr1b* transcript levels accumulated parallel to immunodetectable protein (Fig 1B). This observation confirms results presented previously [8], according to which *mdr1b* mRNA expression is increased dramatically during hepatocyte culture.

To examine the effect of culture duration on mdr1-dependent transport activity, intracellular levels of the fluorescent mdr1-substrate rhodamine 123 achieved after 2 hours of incubation with the dye were compared in hepatocytes cultivated for differing periods of time (1-3 days, Fig. 2). Intracellular dye levels decreased from the first to third day in culture, an observation which is in accordance with a temporal increase in rhodamine 123 extrusion. Furthermore, cells concomitantly incubated with rhodamine 123 and with the chemosensitizer verapamil which is known to interfere with mdr1-dependent drug efflux [29] exhibited higher dye levels. The present data support the conclusion that the temporal increase in P-glycoprotein expression is accompanied by an increase in (a) functional transporter(s) of mdr1 substrates and are in accordance with previous reports describing an increase in *mdr* transcript and P-glycoprotein levels in combination with elevation in efflux of the mdr1 substrate doxorubicin [16, 30].

Supplementation of standard culture medium with 0.16-16 nM EGF led to marked enhancement of time-dependent P-glycoprotein overexpression in cells grown on a collagen matrix (Fig.

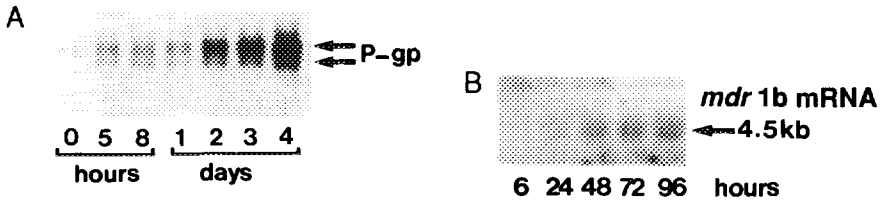

Fig. 1. Time course of mdr expression in primary cultures of rat hepatocytes.
Cells were cultivated as described in "Materials and Methods" for the periods of time indicated.
A. Immunoblot analysis of P-glycoprotein expression in isolated plasma membrane fractions.
B. Northern blot analysis of mRNA expression using *mdr1b*-specific oligonucleotide probe.

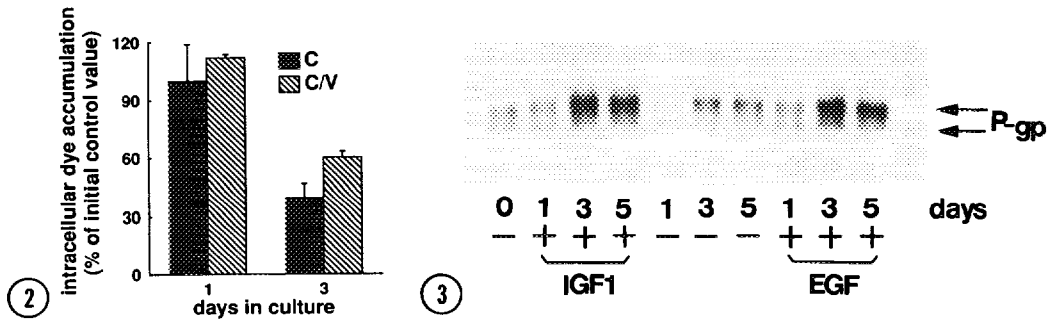

Fig. 2. Intracellular rhodamine 123 accumulation.
Primary hepatocytes were maintained in culture for 1 and 3 days. Subsequent to incubation with rhodamine 123 for 2 hours, intracellular dye levels were determined in n-butanol extracts as described under "Materials and Methods". Values are expressed as percentage of dye levels of control "C" at day 1 of culture. "C" designates cells incubated with 6.5 µM rhodamine, "C/V" cells concomitantly treated with rhodamine 123 and 8 µM verapamil. Data represent mean values ± SD of three independent experiments performed in triplicate.

Fig. 3. Enhancement of P-glycoprotein overexpression in primary rat hepatocytes in the presence of growth factors. Cells were grown in MX-83 medium supplemented with insulin and hydrocortisone in the presence of 16 nM EGF or 130 nM IGF-I for 1-5 days; immunoblot analysis of cytoplasmic membrane fractions.

3). Densitometric analysis of western blot exposures demonstrated that stimulation of P-glycoprotein overexpression was maximal at 8-16 nM EGF (2.4-fold increase in lower molecular weight form and 4-fold increase in higher molecular weight form, Fig. 3); however, enhancement of P-glycoprotein increase was already observed at a concentration of 0.16 nM EGF. Treatment of cells with the growth factor IGF-I also led to an increase in P-glycoprotein overexpression (Fig 3), a maximal effect being exerted by concentrations of 13 nM IGF-I and above. *Mdr1b* mRNA expression increased under EGF in a time-dependent manner parallel to the increase in overexpression of immunodetectable protein (Fig. 4). Hepatocytes cultivated with 130 nM IGF-I for 4 days exhibited *mdr1b* mRNA levels comparable to those in cells grown in the presence of 16 nM EGF for the same period of time.

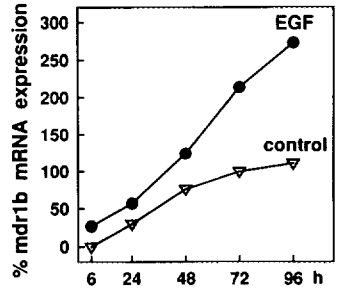

Fig. 4. Increase in *mdr1b* mRNA expression in hepatocytes treated with EGF.
Cells were cultivated in medium containing insulin and hydrocortisone in the presence or absence (control) of 16 nM EGF for up to 96 hours. Data are presented as percentage of expression in control cultures on day 3.

To elucidate whether P-glycoprotein expression under EGF might comprise functionally active mdr1 isoform(s) intracellular rhodamine 123 accumulation was determined. EGF led to enhancement of the temporal decrease in intracellular dye levels; a maximal difference in relative rhodamine retention in cells treated with EGF as opposed to control cells was observed on the third day of culture (Fig. 5), amounting to approximately 40% lower relative retention in respect to control dye levels on the same day of culture. EGF-dependent decrease in dye retention on the third day of culture was inhibited by 8 μM verapamil (Fig. 5), supporting the conclusion that EGF caused induction of functional transporter(s) eliciting rhodamine extrusion and exhibiting sensitivity to verapamil. Nevertheless, the possibility that other P-glycoprotein-related transporters mediating transport of mdr1 substrates (such as the recently characterized product of the *mrp* gene [31]) might contribute to dye transport in EGF-treated cells cannot be ruled out. Stimulation of transport activity by EGF was by far less marked than the influence on expression of immunodetectable P-glycoprotein. A possible explanation for this discrepancy may be that P-glycoprotein induced by EGF might also comprise the mdr2 isoform which does not mediate transport of known mdr1 substrates. Mdr2 expression has been shown to increase during experimentally-induced liver regeneration [32], and EGF receptor activation, elicited by the ligand TGF-α, plays a role in mediating liver regeneration [17].

EGF in concentrations of 1-10nM acts as a potent inducer of DNA synthesis in hepatocytes [20]; therefore, the question may be raised whether P-glycoprotein overexpression might be linked to cell cycle progression. However, in comparison of cultures grown in presence of 1 μM insulin to cells raised in the absence of insulin no increase in basal P-glycoprotein overexpression was observed under insulin (data not shown) although stimulation of DNA synthesis in adult rat

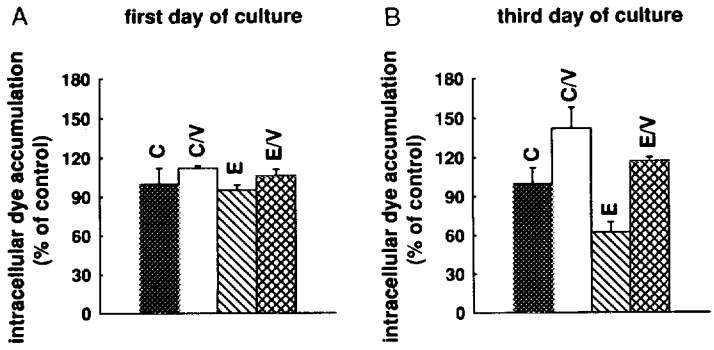

Fig. 5. Influence of EGF on intracellular rhodamine 123 accumulation as a function of culture duration. Hepatocytes were pretreated with 16 nM EGF ("E") or without EGF ("C") for 1-3 days under standard culture conditions. Intracellular rhodamine 123 accumulation was determined according to "Materials and Methods". Cultures concomitantly incubated with 6.5 μM rhodamine 123 and 8 μM verapamil are referred to as "C/V" and "E/V". To define EGF-dependent decrease in dye accumulation in relation to basal decrease in control cultures, relative dye accumulation is expressed as ratio of dye levels in extracts of EGF-treated cultures to dye levels in extracts of cultures grown in absence of EGF for the same period of time. Data represent mean values ± SD of three independent experiments performed in triplicate.

hepatocytes has been shown to occur *in vitro* at insulin concentrations as low as 17 nM [33]. Therefore, P-glycoprotein overexpression seems not to be associated with hepatocyte DNA synthesis alone. Nevertheless, enhancement of P-glycoprotein expression under influence of EGF and IGF-I indicates that signal transduction pathways originating in activation of distinct growth factor receptors may converge in respect to regulation of P-glycoprotein expression and suggest that hepatotrophic growth factors may act as further endogenous modulators of hepatic P-glycoprotein expression.

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