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Influence of isolation procedure, extracellular matrix and dexamethasone on the regulation of membrane transporters gene expression in rat hepatocytes

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Abstract

The influence of the isolation procedure of hepatocytes, extracellular matrix (ECM) configuration and incubation medium supplementation by dexamethasone (DEX) on the cell morphology and on the gene expression of membrane transporters was examined in rat hepatocytes. The mRNA levels were determined using oligonucleotide microarrays, in liver, in suspension and in primary culture in monolayer (CPC), and in collagen gels sandwich (SPC) in absence and presence of DEX (100 and 1000 nM). The results indicated pronounced morphological differences between CPC and SPC in response to DEX demonstrating that the hepatocytes re-formed, as *in vivo*, multicellular arrays with extensive bile canalicular network only in SPC in presence of DEX. The mRNA levels of membrane transporters were not affected significantly during isolation procedure. However, plating hepatocytes in CPC resulted in a decrease of major basolateral transporters mRNA level whereas mRNA levels of mdr1b and mrp3 were increased (>100-fold). Similar observations were made in SPC in the absence of DEX demonstrating that the ECM configuration alone did not play a critical role in the regulation of membrane transporters. However, adding DEX to the incubation medium in SPC resulted in an up-regulation of mdr2, oatp2 and mrp2 in a concentration-dependent way for the two latter genes, whereas mdr1b and mrp3 expression were maintained to their baseline liver levels. These data suggested therefore that the combination of ECM and DEX supplementation is essential for the formation of the bile canalicular network and is a determinant factor in the regulation of membrane transporters in cultured rat hepatocytes.

Keywords: Sandwich culture; Bile canaliculi; Regulation; Chip; Extracellular matrix; Transport

1. Introduction

In vivo, hepatocytes are polarized and express different transport systems located at their basolateral, lateral and canalicular domains. On the basolateral membrane, several active transporters such as Na^+-taurocholic acid transporting polypeptide (ntcp), organic anion transporting polypeptide (oatp1 and oatp2), and organic cation transporter (oct1) are expressed and play an important role in the Na^+-dependent uptake of bile acids, Na^+-independent uptake of organic anions and organic cations, respectively [1,2]. In addition, bile acids are taken up in a Na^+-independent way by the liver-specific rat organic anion transporter rlst1 [3]. The bile canalicular membrane of the mammalian hepa-

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Abbreviations: mRNA, messenger RNA; CPC, conventional primary culture; SPC, sandwich primary culture; DEX, Dexamethasone; mdr, multidrug resistance; ntcp, Na^+-taurocholic acid transporting polypeptide; oatp, organic anion transport polypeptide; oct, organic cation transporter; spgp, sister of P-glycoprotein; P-gp, P-glycoprotein; cbsep, canalicular bile salt export pump (rat); ATP, adenosine triphosphate; ABC, ATP-binding cassette; HEPES, *N*-2-hydroxy-ethylpiperazine-*N*′-2-ethanesulfonic acid; ECM, extracellular matrix; OD, optical density; PM, present match; MM, mismatch; RFU, relative fluorescence intensity; GETP, gene expression-time profile; SU, suspension; LI, liver.

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tocyte contains several primary active transporters that couple ATP-hydrolysis to the transport of specific substrates into the bile canaliculus [4]. These transmembrane transporters are members of the ATP-binding cassette (ABC) transporters and currently include the multidrug resistance associated protein transporter (mrp2 or cmoat), the P-glycoproteins (mdr1, mdr2) and the sister P-gp (spgp) or cbsep that have been shown to act as export pumps for their ligands into bile [2]. The mdr1 sub-family (P-glycoprotein), encoded by two different genes in rodent (mdr1a and mdr1b), is thought to be responsible for the excretion of cationic and neutral amphipathic compounds whereas mdr2 acts as an export pump of phospholipids [5,6]. The canalicular multispecific organic anion transporter (cmoat or mrp2) is largely involved in the biliary excretion of many organic anions including conjugated xenobiotics [7]. Some other transporters identified at the basolateral membrane with similar substrate specificity as the canalicular mrp2 can also act as efflux pumps by exporting drugs from cytosol to blood [6,8–11]. In fact, at least three new members of the mrp family that have been identified, mrp1, mrp3 and mrp6 are expressed in rat liver. Mrp6 is localized at the lateral and, to a lesser extent at the canalicular membrane of hepatocyte and is supposed to fulfill a housekeeping transport function involved in regulation of para- or trans-cellular solute movement from blood to bile [10]. The localization of mrp3 is controversial and needs further investigations. It is thought to be present at the basolateral membrane and/or at the canalicular membrane of the hepatocytes [9,12]. Another member of this transporter family (mrp1) was identified and present at a very low level in rat liver at the lateral membrane [13].

The regulation of sinusoidal uptake and canalicular secretion occurs at different levels. It was shown that the regulation is dependent on the physiological environment, osmolarity, transporter gene expression and transporter degradation [14–16]. On a short-term basis, the level of substrate availability, the covalent modification of transporters, and their regulated exocytic insertion into or retrieval from the membrane may also contribute to their regulation [17]. It became evident that the expression of some of these proteins, particularly involved in transport processes, is extensively regulated *in vivo*. Therefore, the secretory functions of bile acids and drugs may be hormonally modulated by either vesicle-mediated retrieval or insertion of transport proteins into the canalicular domain, thus regulating their surface density [18].

Strong efforts are made to mimic the *in vivo* situation in various *in vitro* models. Several strategies have been pursued to maintain the morphology and the liver-specific properties of hepatocytes. After isolation from liver, the hepatocytes can be maintained in different extracellular matrices (ECM) configuration (single or double collagen matrices, Matrigel, Vitrogen) in customized culture media supplemented with different nutrients including mainly glucocorticoids, growth factors, insulin or hydrocortisone. Nevertheless, ''de-differentiation'' is well known to occur in primary monolayer culture, where hepatocytes lose many of their specific properties such as reduced synthesis of serum proteins; a progressive fall in levels of glucose-6-phosphatase; a decrease in cytochromes P450, NADPH cytochrome P450 reductase [19,20]. In contrast, it has been shown that the hepatocytes cultured between two layers of hydrated collagen in a medium containing dexamethasone (DEX), a synthetic glucocorticoid, retrieve their membrane polarity [21–23], a variety of cell functions and especially their excretion capacity after 4 days [24].

Various liver functions have been demonstrated to be also strongly modulated both *in vivo* and *in vitro* by soluble factors, and for instance corticosteroid hormones. There is a general consensus that glucocorticoids markedly improve the attachment, survival, morphology, and overall performance of hepatocytes seeded on single substrata [25,26]. As suggested by the effects of DEX on the overall pattern of protein synthesis, glucocorticoids directly influence a wide range of activities in culture. DEX has been found to increase fibronectin secretion, induce tyrosine aminotransferases, promote an ordered arrangement of the cytoskeleton, enhance gap junction expression and function, regulate the P-gp expression, support P450 activity, and curtail the decrease in protein synthesis observed in hepatocytes during the initial 24 hr [16,25–36]. The formation of bile canalicular networks is enhanced in the presence of dexamethasone, especially in conjunction with an overlay of ECM [33,34]. Recent data suggest that the ECM plays an important role in the maintenance of differentiated characteristics of primary hepatocytes by inducing the transcription of liver-specific genes and, also, by destabilizing the mRNAs of ubiquitously-expressed genes [37].

Taken together, the extracellular matrix addition and/or the medium composition may have a significant impact on expression of the different transport proteins [38–40]. However, there is still a lack of fundamental understanding of all necessary factors to culture and maintain differentiated hepatocytes. The present study was therefore designed to investigate (i) the impact of the isolation process, (ii) the influence of the configuration of the extracellular matrix, and (iii) the effect of dexamethasone treatment on the cell morphology and on the expression at the mRNA level of genes encoding membrane-specific transport proteins in hepatocytes. The gene expression of the different liver-specific sinusoidal, lateral and canalicular transport proteins was therefore determined using rat GeneChip®, a high density oligonucleotide microarrays, in male rat liver, in suspension of freshly isolated hepatocytes and at different culture stage under conventional and sandwich configurations. In order to evaluate the impact of dexamethasone, the mRNA

levels of specific hepatic transporters was evaluated in hepatocytes in sandwich configuration in response to dexamethasone.

2. Materials and methods

2.1. Experimental design

2.1.1. Design of study

The mRNA levels were measured in liver tissue, in freshly isolated hepatocytes in suspension ($T = 0$ hr), and in hepatocytes in primary culture under conventional (CPC) and sandwich (SPC) configurations. In comparison to the baseline level represented by the expression in liver tissue, the mRNA levels in suspension represents the expression after the isolation of hepatocytes (~30 min) from liver tissue by collagenase perfusion. In CPC, the freshly isolated hepatocytes were seeded on a single solid collagen matrix in BIOCOAT® plates and mRNA levels were measured after cell attachment ($T = 3-4$ hr) and after 8, 20 and 44 hr of culturing. In SPC, cells were seeded between two layers of collagen gel in absence and presence of 100 and 1000 nM of DEX. The gene expression was measured at the same time points as in CPC and also after 68 and 92 hr. The influence of the extracellular matrix was evaluated by the comparison of the gene expression-time profiles (GETP) in CPC vs. SPC in absence of DEX. The impact of DEX on the gene expression of transport proteins was studied by comparison of the GETP in SPC without and with 100 and 1000 nM of DEX in the incubation medium.

2.1.2. Genes of interests

All selected genes are referenced in Table 1. Genes are classified by the membrane domain location of the encoded transport proteins. For transport proteins located at the sinusoidal membrane and responsible for the uptake and efflux processes, the genes encoding oatp1, oatp2, ntcp, oct1, oat-2 and mrp3 were selected. At lateral membrane, genes encoding mrp1 and mrp6 were of special interest. For proteins involved in hepatobiliary transport and located at the canalicular membrane we selected genes encoding mdr1a, mdr1b, mdr2, spgp (bsep) and mrp2 (cmoat).

2.2. Experimental procedures

2.2.1. Materials

2.2.1.1. Chemicals. Ca^{2+}-free Krebs–Henseleit bicarbonate buffer, insulin, glutamine, ethylene glycol-bis(beta-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetra acetic acid (EGTA), Glucose, *N*-2-hydroxy-ethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), new-born calf serum, DMEM 10-fold, Williams' E medium without L-glutamine and without phenol red, Leibovitz L-15 medium, Percoll solution and insulin were all purchased at Sigma. Collagenase (CLS2 LS04176) was obtained from Worthington Biochemical Corporation. $CaCl_2 \cdot 2H_2O$ was purchased from Fluka and the Erythrosin B disodium salt from Aldrich. Rat tail collagen type I in solution at 4.4 mg/L was purchased from Collaborative Biomedical Products. Streptomycin/Penicillin and SSPE buffer (20×) were purchased from GibcoBRL. RNAlater solution was purchased from Ambion Inc., RNAzol extraction solution from Biotecx Laboratories Inc. and CIA buffer, DIPS solution, SEWS solution, RNA-free water were purchased from Bio101.

2.2.1.2. Microarrays. The rat genome U34A Affymetrix GeneChip® array (reference number: 900249) containing 8800 sequences from Affymetrix (Santa Clara, CA, USA) was used.

Table 1
References of specific rat hepatocyte membrane transport proteins and corresponding coding genes

Gene product	Gene name	Protein accession number	Gene accession number	Location
ntcp	SLC10A1	P26435	M77479	Basolateral
oatp1	SLC21A1	P46720	L19031	Basolateral
oatp2	SLC21A5	O35913	U88036	Basolateral
oatp4	SLC21A10	Q9JHF6	AJ271682; CAB92299	Basolateral?
oct1	SLC22A1	Q63089	X78855	Basolateral
oat-2	SLC22A7	Q63314	L27651; L30107	Basolateral
mdr1a	PGY1A	Q9JK64	AF257746; S66618	Canalicular
mdr1b	ABCB1; PGY1B	P43245; X61103	M62425; M81855	Canalicular
mdr2	PGY3	Q08201; L15079	L15079	Canalicular
bsep	ABCB11	O70127	U69487; AF010597	Canalicular
mrp2	ABCC2	Q63120	L49379; X96393; D86086	Canalicular
mrp6	ABCC6	O88269; U73038	AB010466; U73038	Lateral? Canalicular?
mrp3	ABCC3	O88563	AF072816; AB010467	Basolateral?
mrp1	ABCC1	Q9JHS0	AJ277881	Lateral

The proteins and genes references were taken from SwissProt® and EMBL® databases. The corresponding oligonucleotide was obtained by a blast of gene sequence against the Roche Affymetrix® oligonucleotide sequence database. The specificity of oligonucleotide sequence was evaluated using Serenis® software tool.

2.2.1.3. Animals. The animal study was carried out with male Wistar rats weighing approximately 200–300 g, and supplied by BRL, Biological Research Laboratories Ltd. They were kept under routine laboratory conditions, received standard laboratory chow and had free access to food and water. Permissions for animal studies were obtained from the local regulatory agencies, and all study protocols were in compliance with the federal guidelines.

2.2.2. Methods

2.2.2.1. Isolation procedure of hepatocytes. The rats were anesthetized by intraperitoneal administration of a physiologic 2% solution of 90 mg/kg ketamine and 10 mg/kg xylazin. The hepatocytes were isolated from rats according to a modified two-step collagenase perfusion method [41]. Briefly, the liver was removed and perfused *ex situ* in a pre-warmed chamberglass with oxygenated Ca^{2+}-free Krebs–Henseleit bicarbonate buffer containing 5.5 mM glucose for 10 min at 37° followed by perfusion with Krebs–Henseleit bicarbonate buffer containing collagenase type I (0.5 mg/mL) for approximately 10 min. The hepatic capsule was removed with forceps. The hepatocytes were released in L-15 medium and filtered through a sterile nylon mesh (60 μm). The hepatocyte suspensions were centrifuged three times at 50 *g* for 3 min. The cell pellet was resuspended in 25 mL L-15 medium and an equal volume of 90% isotonic Percoll solution (pH 7.4) to remove non viable cells; the resulting cell suspension was centrifuged at 50 *g* for 10 min. The pellet was resuspended in 50 mL L-15 and the cell suspensions were combined into one tube followed by centrifugation at 50 *g* for 3 min. Hepatocyte viability was determined by erythrosin-B exclusion. Only hepatocytes preparations with viability greater than 85% were used for further studies.

2.2.2.2. Conventional primary culture (CPC). Freshly prepared hepatocytes were seeded on single solid collagen matrix on precoated BIOCOAT® 6-well plates (Reference 354400, Falcon, Becton Dickinson). Cells (1×10^6 cells per well) were cultured for 3 hr in a humidified chamber maintained at 37°, 5% CO_2 in attachment medium composed of Williams' E medium supplemented with 10% fetal calf serum, 0.5% streptomycin/penicillin, insulin (1.2×10^{-6} M) and glutamine (400×10^{-6} M). After 3 hr attachment, the medium was removed and replaced by an incubation medium corresponding to the attachment medium but supplemented with 10^{-7} M hydrocortisone. The latter medium was changed once a day.

2.2.2.3. Sandwich primary culture (SPC). Hepatocytes (1.4×10^6 cells/mL per well) were seeded in precoated dishes (6-well plate, Falcon). The collagen precoating was performed using physiological pH-adjusted (pH 7.4) rat tail collagen type I solution diluted at 3 mg/mL with DMEM medium 10-fold. A total amount of 100 μL of ice-cold collagen solution was added and spread in the well. Coated plates were then stored in a sterile incubator at 37° for 45 min to allow the collagen to gel. After 3 hr attachment in a humidified incubator at 37°, 5% CO_2, the medium was aspirated and neutralized collagen solution (∼100 μL) was added on the cell monolayer. Plates were returned to the incubator for 30–40 min to allow the collagen to gel after which long-term cell culture medium was added. This medium was changed on a daily basis until the fourth day after seeding. The long-term cell culture medium was prepared by adding 25 mL fetal calf serum, 2.5 mL streptomycin/penicillin, 1 mL of 200 mM glutamine, 100 μL of 0.24 mg/mL hydrocortisone, 0.2 mL of 10 mg/mL insulin and dexamethasone (100 or 1000 nM) to 500 mL of Williams' E medium.

2.2.2.4. Isolation of total RNA from liver tissue, isolated hepatocytes in suspension and cultured cells. The liver pieces (N = 4) were rapidly soaked into 50 mL falcon tubes containing 20 mL RNA-Later, and placed on ice. The samples were kept for a day at 4°, and then stored at −20°. Approximately 100 mg tissue in RNA-Later or frozen tissue and 1.2 mL RNAzol were combined in a cold FastRNA tube green (Bio 101; FastPrep). From hepatocytes suspension, an aliquot of 2.8×10^6 hepatocytes were taken up from suspension, placed in 1 mL of RNAzol and stored at −20° before mRNA extraction. For isolation of RNA from cells in culture, medium was removed after the indicated culture periods (at 3, 8, 20 and 44 hr for CPC and 3, 8, 20, 44, 68 and 92 hr for SPC). Then the cell layer was carefully washed three times with 1 mL of prewarmed (37°) PBS. PBS was removed and 1 mL of cold RNAzol solution was added in the first well. The cells from two wells were scraped, pooled and stored at −80° for total RNA extraction. Total RNA was extracted using BIO101 kit according to manufacturer instructions. An aliquot (3 μL) was checked on 1% agarose gel and quantified by optical density (OD) measurement.

2.2.2.5. Gene expression determination. The validity of the approach to measure the relative mRNA transcripts abundance using oligonucleotide microarrays was demonstrated by several investigators in bacteria [42] but also recently in cultured hepatocytes [43]. The mRNA that is isolated from the tissue or from cultured hepatocytes is converted to fluorescently labeled cDNA and used to hybridize the microarray. All expressed genes in the sample studied will bind to one probe set of the array and generate a fluorescent signal with an intensity function of the mRNA abundance. The sequences of the genes of interest were found in EMBL database. The corresponding probe sets were found by a comparison of the EMBL gene sequence against the Affymetrix oligonucleotide sequence database. This procedure was done using the BLAST® program, a similarity search program designed to explore all of the available sequence databases regardless of whether the query is protein or DNA. For clarity, the gene name was italicized in comparison to the encoded protein name

which is not italicized (example: gene, *mdr1b*; protein, mdr1b).

2.2.2.6. Affymetrix GeneChip® arrays processing. cRNA labeling, hybridization and washing were performed using standard procedures as previously described [44].

2.2.2.7. Microarray data analysis. Microarrays were scanned at 570 nm, 3 μm resolution with a gene chip scanner (Affymetrix) and analyzed as previously described [45]. For quantification of relative transcript abundance, the average difference value (Avg Diff) was used. The signal intensity for each gene was calculated as the average intensity difference represented by ((PM−MM)/(number of probe pairs)). All chips were normalized by the mean of the total sums of Avg Diff values across all chips used in the experiment allowing a comparison between conditions. An Avg Diff level lower than 200 fluorescence units was considered below the limit of accurate quantification based on extensive quality control experiments performed in house. Above 200 fluorescence units, based on our in house validation dataset, any gene having an absolute ratio or change factor >2, was considered significantly differentially regulated.

2.2.2.8. Normal light microscopy. Pictures were taken under an inverted normal light Nikon® microscope (magnification 20×, filter set Ph2) at day 2 for the CPC and at day 5 for the SPC.

3. Results

For direct confirmation of the temporal transcript changes obtained from oligonucleotide microarrays data analysis, the data obtained in this study were compared to literature data obtained by RT-PCR for well characterized genes. We chose genes with varying expression patterns for confirmation analysis to ensure that differences in both induced and repressed transcripts could be reliably reproduced. As example it has been reported in the literature that the *CYP3A* was induced by DEX, that the *mdr1b* was highly up-regulated in CPC and that major *CYP450*s were down-regulated in CPC. In this sense, all these prototypical patterns were confirmed by the expression trends observed via microarrays analysis.

3.1. Impact of the isolation procedure: comparison between the expression of genes encoding liver plasma membrane transporters in liver (baseline level) and in freshly isolated hepatocytes in suspension

The change factor characterized by the ratio between the gene expression in suspension compared to those in liver (ratio SU/LI in Table 2) was below 2 ($0.5 < SU/LI < 2$) for all selected genes with the highest value for the gene encoding ntcp which was up regulated to 1.60 times the *in vivo* level. Therefore, as shown in Figs. 1–3 (conditions: Liver vs. Suspension), the data revealed that isolation procedure and cell attachment does not influence critically, on a short-term basis, the expression of genes encoding transport proteins located on plasma membranes.

3.2. Impact of the extracellular matrix (ECM) configuration: comparison of GETP in CPC and SPC without DEX

The expression of almost all genes encoding basolateral transport proteins except the gene encoding mrp3 (an ATP-dependent transporter), showed a marked decrease from their initial 3-hr value to reach the detection limit after

Table 2
Impact of isolation procedure of hepatocytes on mRNA levels of genes encoding basolateral, lateral and canalicular transporters

Gene product	Liver (N = 4)		Suspension (N = 3)		
	±SD baseline level	C V (%)	±SD	CV (%)	SU/LI
oct1	1586 ± 847	53	2045 ± 227	11	1.3
ntcp	2905 ± 795	27	4771 ± 1900	40	1.6
oatp1	450 ± 214	48	615 ± 393	64	1.4
oatp2	2848 ± 307	11	3156 ± 1352	43	1.1
oat2	312 ± 168	54	252 ± 21	8	0.8
oat2	525 ± 76	15	705 ± 32	5	1.3
mrp1	<100	n.d.	<100	n.d.	n.d.
mrp3	<100	n.d.	<100	n.d.	n.d.
mrp6	543 ± 200	37	429 ± 85	20	0.8
mrp2	657 ± 76	12	834 ± 456	55	1.3
mdr2	228 ± 60	26	279 ± 116	42	1.2
mdr1a	<100	n.d.	<100	n.d.	n.d.
mdr1b	<100	n.d.	<100	n.d.	n.d.
spgp	692 ± 192	28	674 ± 229	34	1.0

The ratio SU/LI represents the change factor between relative mRNA level in suspension and corresponding mRNA level in liver. The mRNA levels in liver and in freshly isolated hepatocytes suspension were evaluated using Affymetrix GeneChip® arrays from liver homogenate (N = 4) and from hepatocytes in suspension (N = 3), respectively. Values with RFU <100 are below quantification limit. Values are given by mean ± SD; n.d., not determined.

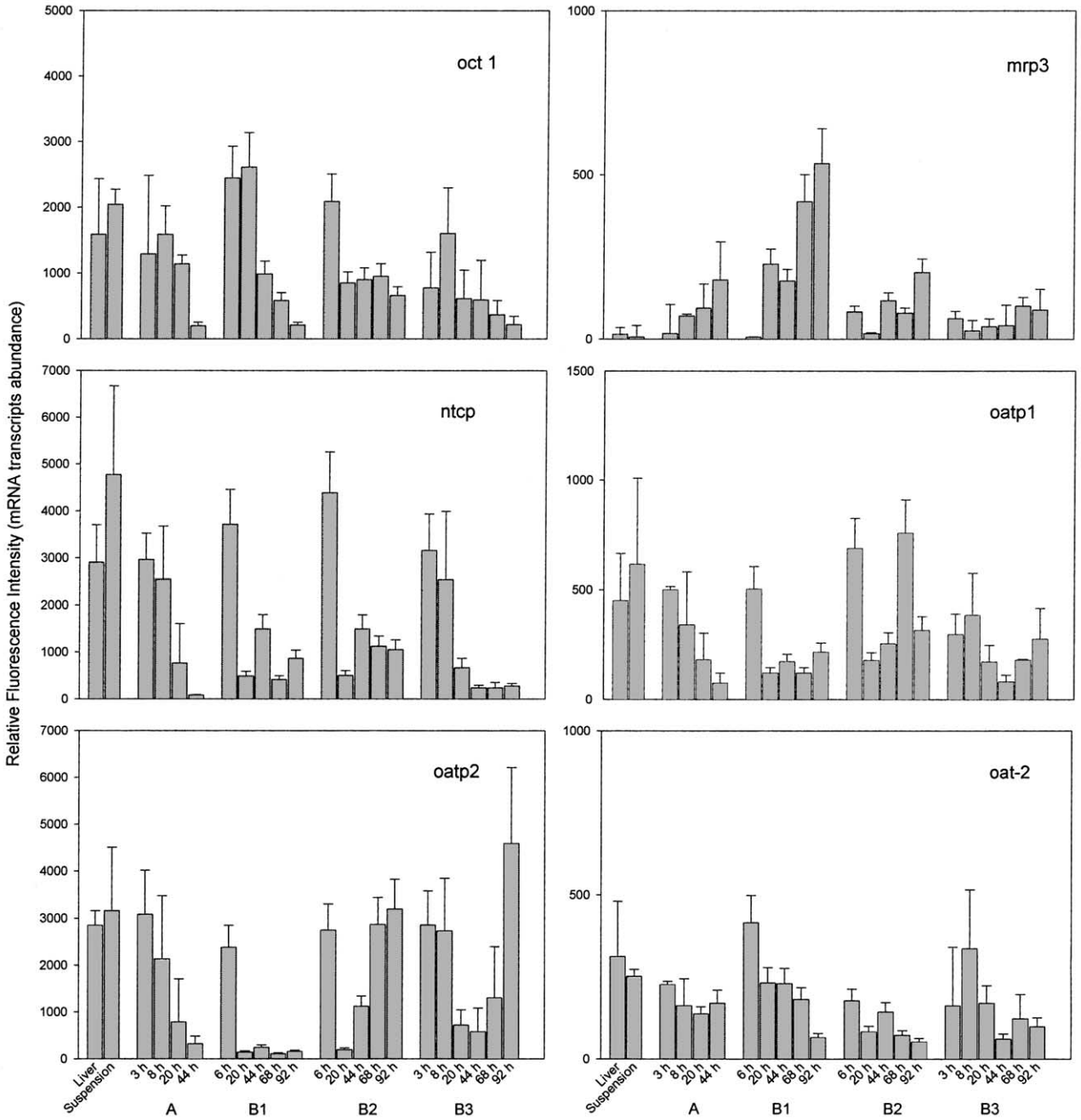

Fig. 1. GETP of basolateral membrane transporters: expression at mRNA level of genes encoding basolateral active transporters in rat hepatocytes (ntcp, oatp1, oatp2, oct1, mrp3 and oat-2) in liver (baseline level; N = 4), in suspension (N = 3) and in primary culture (N = 3) when hepatocytes were plated on single collagen matrix (CPC) (A) and between two layers of collagen gels (SPC) in absence (B1) and in presence of 100 nM (B2) and 1000 nM (B3) of DEX. The mRNA abundance related to the relative fluorescence intensity (RFU) was measured with rat U34A GeneChip®. Data represent the mean and SD of separate experiments.

44 hr. This was observed in CPC and in SPC when DEX was omitted from the incubation medium (Figs. 1–3, conditions: A vs. B1). *Ntcp*, *oatp1*, *oatp2* and *oat-2* mRNA levels were below the detection limit after 20 hr whereas *oct1* mRNA levels seems to better maintained during this culturing period (~1.6-fold change CPC20/CPC3; no change SPC20/SPC3). Between 20 and 92 hr of culture, the *oct1* mRNA levels decreased rapidly to reach the detection limit. The expression of the *mrp3* gene, which was expressed to low levels in the liver and in suspension was strongly up-regulated in both culture system (>100 fold change). These findings showed that an additional ECM (SPC w/o DEX vs. CPC) does not prevent the loss of the gene expression of basolateral transporters responsible for the uptake of drugs.

The *mrp1* gene was not significantly modulated in CPC and in SPC without DEX (Fig. 2). In contrast, the expression of the gene encoding mrp6 was changed under both

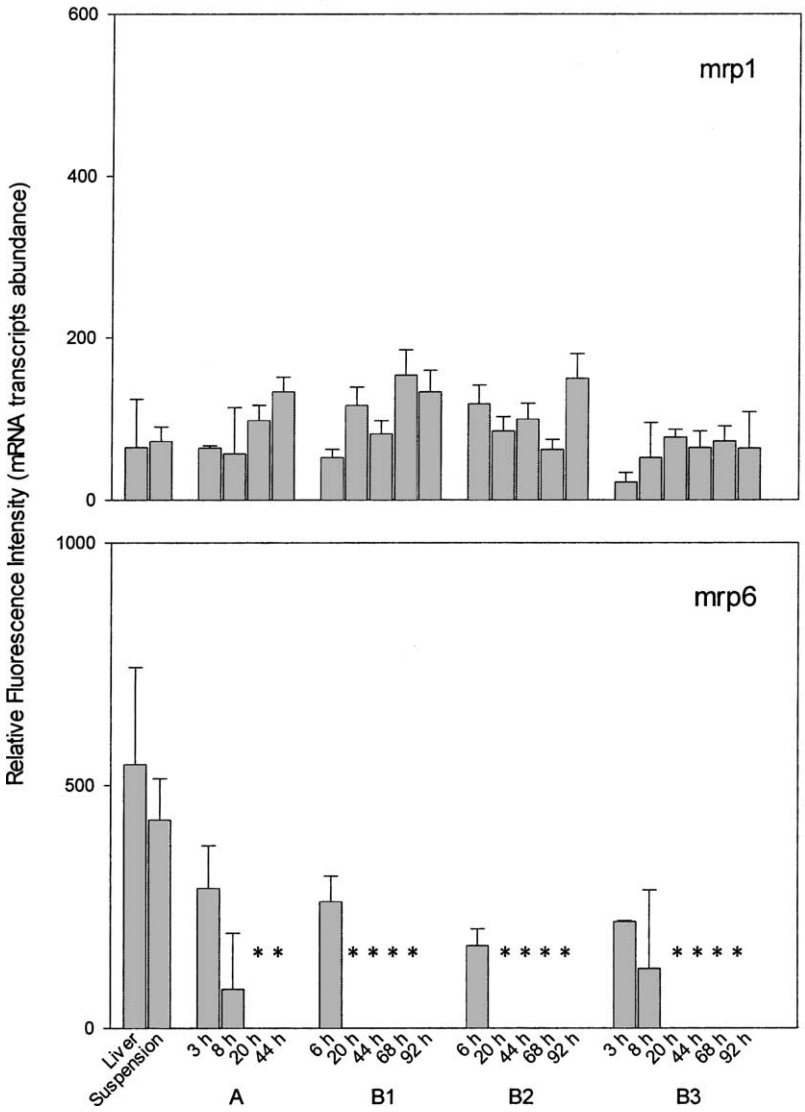

Fig. 2. GETP of lateral membrane transporters: expression at mRNA level of genes encoding lateral (mrp1, mrp6) transporters in rat hepatocytes in liver (N = 4), in suspension (N = 3) and in primary culture (N = 3) when hepatocytes were plated on single collagen matrix (CPC) (A) and between two layers of collagen gels (SPC) in absence (B1) and in presence of 100 nM (B2) and 1000 nM (B3) of DEX. The mRNA abundance related to the RFU was measured with rat U34A GeneChip®. Data represent the mean and SD of separate experiments. The asterisks represent the mRNA transcripts abundance below the limit of quantification.

conditions. The gene expression of *mrp6*, which was relatively highly expressed in the liver, was not expressed in CPC and decreased rapidly in SPC to reach the detection limit after 20 hr of culture. Therefore, the GETPs showed that the top ECM has no significant impact on the regulation pattern of genes encoding lateral membrane transporters.

The *mdr1a* mRNA level was maintained at the baseline level and was therefore not modulated by the ECM but also not modulated by the culture process (Fig. 4, conditions: A vs. B1). The gene encoding the mdr1b isoform was strongly up-regulated in both conditions (>100-fold change) showing no effect of the ECM. The *mdr2* mRNA level decreased gradually after 8 hr in CPC whereas it was maintained during the first 44 hr in SPC (Fig. 4, mdr2, conditions: A vs. B1). The gene encoding the spgp as compared to the 3-hr value was down regulated to approximately 50% of the baseline mRNA level after 44 hr of culture in CPC. In SPC, a first decrease was observed during the 20 hr of culture and then was increased until 44 hr to gradually decline until 92 hr to approximately 60% of liver mRNA level. For mrp2 mRNA levels (Fig. 4), an up-regulation was observed during the first 20 hr in CPC and during the first 44 hr in SPC. Then, the expression decreased to a level comparable to the baseline level after 44 hr in CPC and after 92 hr in SPC. We observed that the expression after 44 hr in SPC was equal to approximately 3.5-fold those in CPC at the same time points. As shown for basolateral and lateral membrane transporters, the regulation pattern in

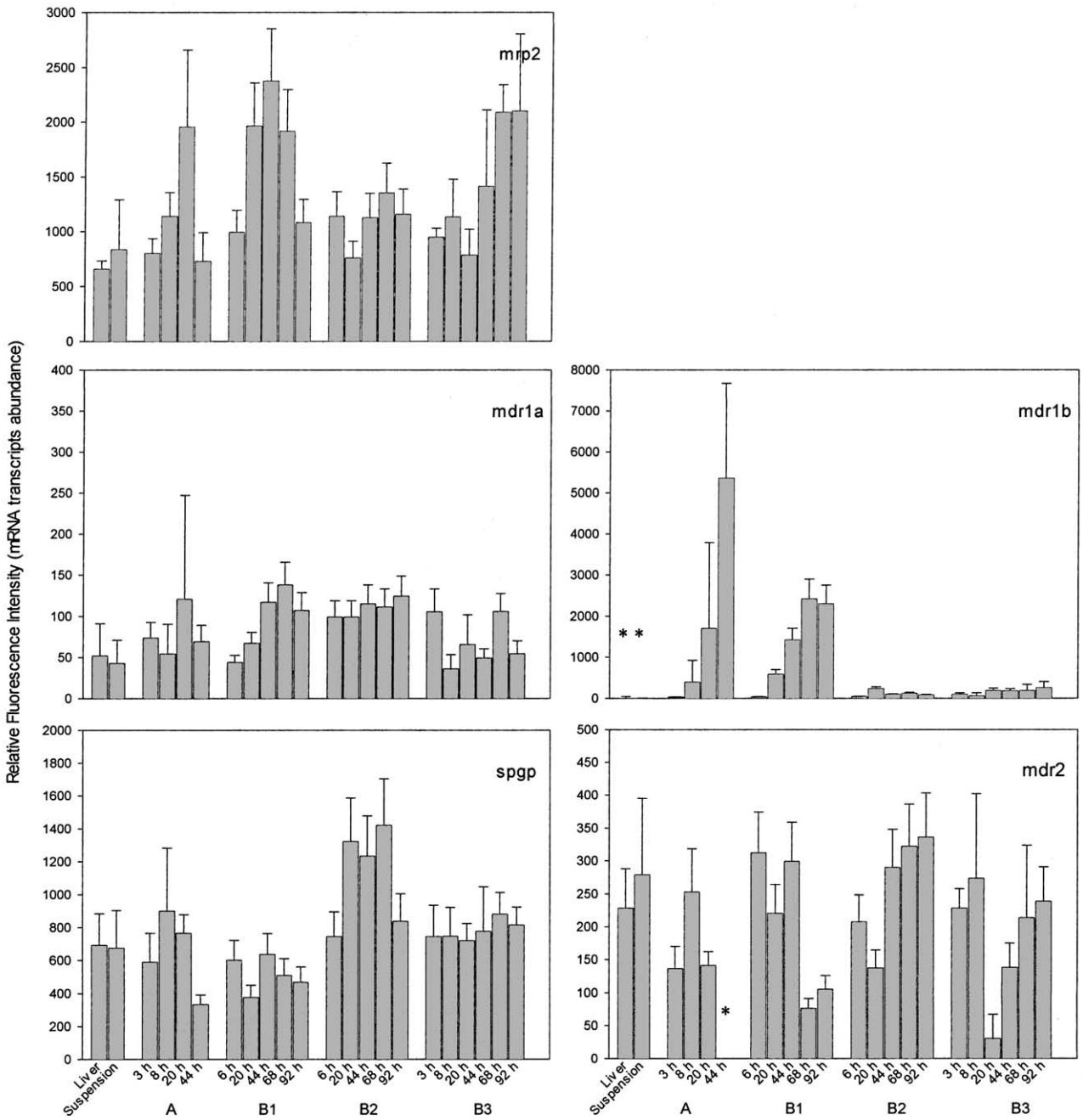

Fig. 3. GETP of canalicular membrane transporters: expression at mRNA level of genes encoding canalicular transporters (mrp2, mdr2, mdr1a, mdr1b and bsep) in rat hepatocytes in liver (N = 4), in suspension (N = 3) and in primary culture (N = 3) when hepatocytes were plated on single collagen matrix (CPC) (A) and between two layers of collagen gels (SPC) in absence (B1) and in presence of 100 nM (B2) and 1000 nM (B3) of DEX. The mRNA abundance related to the RFU was measured with rat U34A GeneChip®. Data represent the mean and SD of separate experiments. The asterisks represent the mRNA transcripts abundance below the limit of quantification.

CPC and SPC without DEX are close, demonstrating that the ECM alone has little effect on the regulation of the genes encoding membrane transporters in cultured hepatocytes.

By comparison of the GETPs of genes encoding membrane transporters in CPC and SPC in absence of DEX, clear observations demonstrated that in our conditions, the ECM does not play a critical role on the regulation pattern of these genes.

3.3. Impact of dexamethasone in SPC: comparison of GETP in SPC in absence (B1) and presence of 100 (B2) and 1000 nM (B3) of DEX

Overall, the results (Figs. 1–3, conditions: B1 vs. B2. vs. B3) revealed different kinds of gene expression modulation by DEX: (i) a concentration dependent up-regulation of *oatp2* and *mrp2* mRNA levels, (ii) a maintenance of the *mdr1b* and *mrp3* mRNA levels at their baseline mRNA

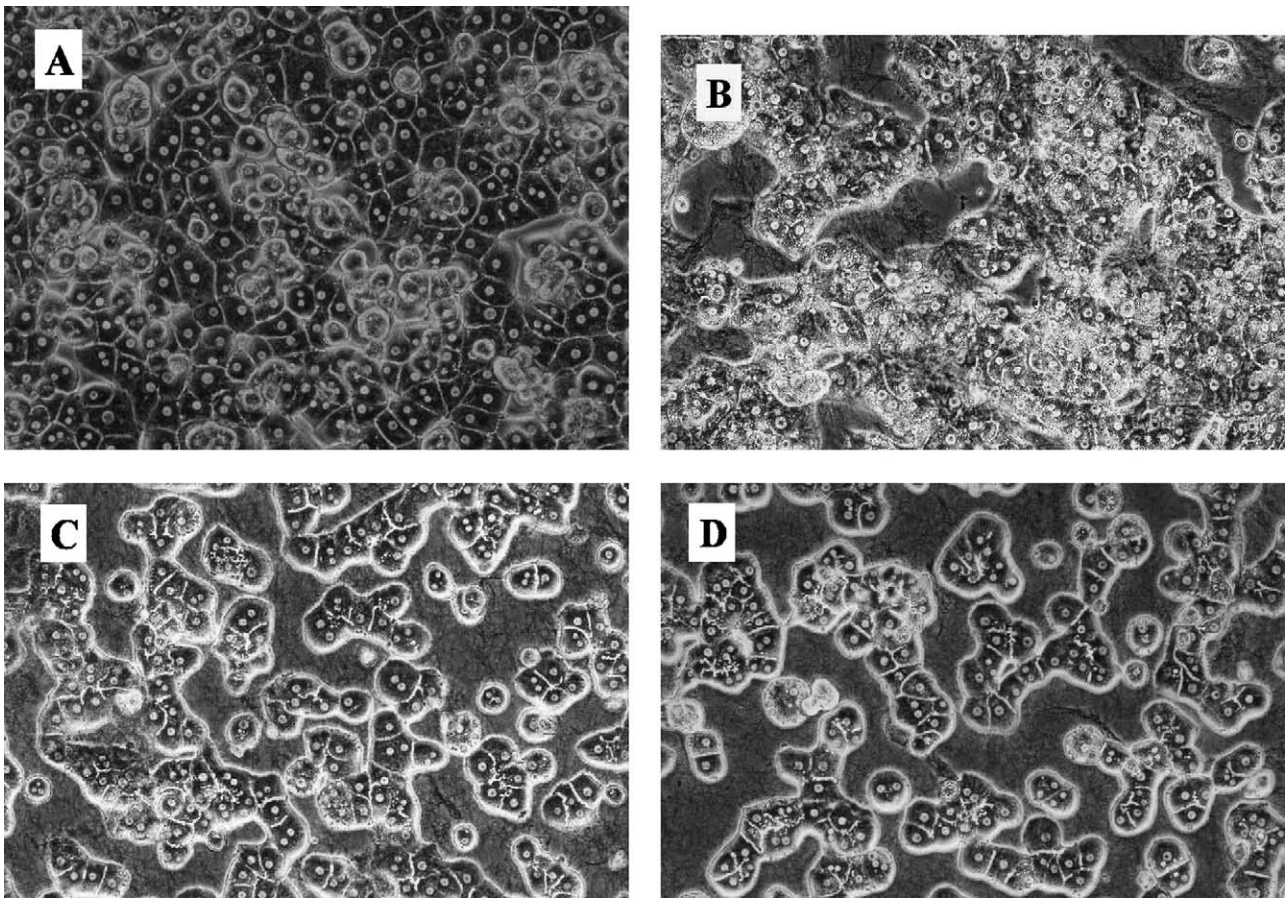

Fig. 4. Impact of DEX on the morphology of hepatocytes. Pictures were taken under an inverted normal light Nikon microscope (magnification 20×, filter set Ph2) at day 2 for the view A and at day 5 for the views B–D. The view A represents a primary culture of hepatocytes on rat tail collagen I BIOCOAT® plate (CPC), 24 hr after cell seeding (1×10^6 cells per well). In B–D, pictures of hepatocytes (1.4×10^6 cells per well) under sandwich configuration (SPC) at day 5 cultured in absence of DEX, presence of 100 and 1000 nM of DEX, respectively.

levels, (iii) a V-shape regulation profile for the genes encoding mdr2, oatp2 and oatp1 in presence of DEX, and (iv) no effect of DEX was observed on the GETPs of genes encoding oct1, ntcp, oat-2, mrp1, and mdr1a.

(i) The *oatp2* gene expression in absence of DEX was first rapidly down regulated with a loss of approximately 95% of the 6 hr-control value after 20 hr of culture. Then, this expression level was maintained until 92 hr (Fig. 1, oatp2; condition B1). By addition of DEX in the incubation medium, no effect was observed on the rapid down-regulation phase but a gradual up-regulation of *oatp2* mRNA levels, which was dependent on the concentration of DEX, was observed after 20 hr of culture period (Fig. 1, oatp2; conditions: B1–3). The maximum of induction was observed after 92 hr of culture to reach approximately the baseline mRNA level in presence of 100 nM of DEX and approximately 1.5 times the baseline mRNA level in presence of 1000 nM of DEX. The *mrp2* mRNA levels in absence of DEX followed a parabolic regulation pattern with a maximum reached at 44 hr (Fig. 3, mrp2; condition B1). In presence of 100 nM of DEX in the incubation medium, the gene was first slightly down regulated during the first 20 hr and then the expression increased to reach a plateau at 68 hr corresponding to a 2-fold baseline mRNA level (Fig. 3, mrp2; condition B2). By increasing the DEX concentration to 1000 nM, the same pattern was observed during the first 44 hr and then the expression increased gradually to reach 3.5-fold the baseline level showing a net concentration effect on the induction phase (Fig. 3, mrp2, condition B3).

(ii) The presence of DEX in the incubation medium suppresses entirely the up-regulation and maintains the gene expression of *mdr1b* and to a lesser extent those of *mrp3* at their baseline mRNA levels (Fig. 1, mrp3; Fig. 4, mdr1b; conditions: B1–3). The *mrp3* mRNA levels in absence of DEX increased with time in sandwich culture. By adding DEX in medium a net stabilization of *mrp3* gene expression was showed without any significant concentration dependence in the concentration range studied. The expression of the gene encoding mdr1b is rapidly and highly induced in absence of DEX to reach a plateau at 68 hr (day 4). In presence of DEX at a concentration up or equal to

100 nM, the expression of *mdr1b* is maintained at normal liver mRNA levels. Interestingly, the *mdr1a* is expressed at a relatively low level at both DEX concentrations, suggesting that *mdr1a* is not regulated by the same mechanism than *mdr1b*.

(iii) Some genes followed in presence of 1000 nM a V-shaped regulation pattern, i.e. an initial down-regulation during the first 20–44 hr and then an up-regulation until 92 hr. This was observed for genes encoding *oatp1*, *oatp2* (Fig. 1), *mdr2* and *mrp2* (Fig. 3). No significant concentration dependent-effect of DEX was observed on the expression of genes encoding *oct1*, *ntcp*, *oatp1*, *oat2*, *mdr2*, *mdr1a*, *spgp*, *mrp1* and *mrp6*.

3.4. Impact of DEX on the morphology of the hepatocytes in CPC and SPC

Freshly isolated rat hepatocytes from the same batch were cultured for 5 days after seeding in CPC and in SPC between two layers of collagen gel in medium without (Fig. 4B) or with 100 and 1000 nM of DEX (Fig. 4C and D). Photographs of cells in CPC were taken after 20 hr (Fig. 4A) and after 96 hr (Fig. 4B–D) under SPC. No pictures of CPC after 3 days were taken due to the rapid degradation of the cell layer. Pronounced morphological differences were observed among the various culture conditions employed. On simple collagen substratum (CPC), cells characterized by their polygonal structure spread, flattened and reached confluence.

The cells maintained in SPC in absence of DEX were confluent with little translucent network but cell borders were not well defined (Fig. 4B). In presence of DEX, the hepatocytes have numerous microvilli and are cuboidal and cluster dynamically (between days 2 and 3) into cords reminiscent of hepatic trabeculae. Their subcellular organelles have normal morphology, and specialized junctions and bile canaliculi formed within the membranes of adjacent cells. The bile canaliculae appeared wider and cells re-formed dynamically multicellular arrays.

By comparison of pictures of CPC (Fig. 4A) and SPC (Fig. 4D), the cell density in SPC appears lower than in CPC. Based on protein content determination in each culture system (data not shown), the cell density at day 5 is higher in SPC than in CPC and close to those observed after cell seeding. This observation is only due to the re-establishment of the three-dimensional morphology in SPC. Consequently, the surface of plasma membrane in contact to the bottom matrix in SPC is lower than in CPC in which the hepatocytes spread and flattened. The hepatocytes in SPC also synthesize fibrin meshworks between cells. No distinct difference in cell aspect was observed between hepatocytes cultured in presence of 100 or 1000 nM of DEX. Taken together, these morphological data suggest that these hepatocytes are re-polarized and generally have an appearance very similar to parenchymal cells in the liver.

4. Discussion

After isolation by collagenase perfusion, the tight junctions between adjacent hepatocytes are destroyed, which lead consequently to the loss of the polarity. Transport proteins may be dynamically redistributed to the spheroid membrane surface of the hepatocyte or internalized into the cell to be finally degraded. Therefore, the rapid de-differentiation of cultured rat hepatocytes may be interpreted as a consequence of the loss of the *in vivo* physiological environment, which contributes to their complex regulation. In fact, after isolation, the hepatocytes should re-adapt to a new environment by modulating their morphology and most of their specific functions. Therefore, it may also result from morphological or transcriptional changes occurring after cell isolation due to the isolation procedure or due to the conditions of culture.

In this study, we have first characterized the effects of the isolation procedure by collagenase on the differential mRNA levels of genes encoding plasma membrane transporters in freshly isolated suspension as compared to baseline liver levels. This to describe if the decreased functionality of hepatocytes in culture may be explained by an irreversible decrease of transcription of these genes. The analysis of transcriptional data revealed for major genes encoding transport proteins that the mRNA levels measured after cell attachment does not differ significantly from baseline level. These results provide evidence that the isolation procedure and the storage time period before cell attachment, does not influence critically, on a short-term basis, the expression of genes encoding transport proteins located on plasma membranes.

In order to estimate the impact of the ECM in culture, freshly isolated hepatocytes were seeded under CPC and SPC in the absence of DEX. In both systems, an extensive down-regulation of basolateral transporters mRNA levels was observed over time, showing that the addition of an ECM overlay could not prevent the down-regulation of basolateral transporters observed in CPC. The decrease was particularly rapid in CPC for *ntcp*, *oct1*, *oat2*, *oatp1* and *oatp2* genes as shown earlier for genes encoding ntcp and oatp2 [46,47]. The down-regulation of *ntcp* mRNA level, one of the principal transporter responsible for the uptake of bile acids, was also shown at the protein level, as well as at the functional level, where a great impact on overall taurocholic acid uptake in primary culture of rat hepatocytes was observed [47]. The *mdr1b* gene, which encode the transport protein located at canalicular membrane and which is responsible of the excretion of organic cations, was strongly up-regulated in both culture systems over a 44-hr period whereas *mdr1a* was remaining constant. These results, which confirmed those from other investigators [6,48–50], indicate that the two rodent mdr1 genes are differentially regulated, and demonstrate that the addition of an ECM could not prevent the up-regulation of the mdr1b gene. The regulation pattern of *mrp2* gene

follows a bell-shaped profile with a maximum of expression between 20 and 44 hr of culture in both systems and no effect of the ECM was observed. This pattern in CPC was confirmed in a recent study where the decrease of *mrp2* mRNA levels after 44 hr was parallel to the mrp2 protein levels [47]. Finally, the gene encoding the basolateral transporter mrp3 was up regulated in both CPC and SPC systems and therefore not affected by the presence of the type of ECM. Globally, when the hepatocytes were maintained in CPC or in SPC in absence of DEX, the results showed that the genes encoding basolateral transporters which are responsible of the uptake of drugs from blood into the cell were down regulated (*oatp*, *oct*, *ntcp*) and that the genes encoding transporters playing a role in export of drugs from the cell to the outside (*mdr1b*, *mrp3*) were up regulated. Such modulations was also observed during cholestasis and in mrp2 deficient mutant rat liver [8] demonstrating that hepatocytes in these configurations protect them by decreasing the basolateral uptake activity and by expressing systems (mdrb, mrp3), which may compensate the loss of endogenous excretory functionality of major transporters over time in culture.

The morphological study using hepatocytes in sandwich culture in presence of DEX showed that hepatocytes in this configuration re-established dynamically, between days 2 and 3 after cell seeding, a bile canalicular network between adjacent cells to reformed at day 5 pseudo sinusoids as shown in acinus, *in vivo* in liver. Several confocal microscopic studies realized in SPC demonstrated that the three membrane domains are distinct after 5 days of culture when the culture medium was supplemented by DEX [21,51,52]. In addition, studies with fluorescent substrates of canalicular transporters, such as CMFDA for mrp2 and Rhodamine 123 for mdr1, confirmed the localization of the proteins at the specific membrane domains. From this study, we demonstrated by normal microscopy that DEX in combination with an extracellular matrix play a critical role in the cell re-polarization. At mRNA levels, it has been shown in primary culture of hepatocytes that glucocorticoids are involved in the *mrp2* [53], *spgp* [16] and *mdr1* [36,54] gene regulation processes, all encoding transporters which are located at the canalicular membrane of the rat hepatocyte. Knowing that the major morphological changes occurred between days 1 and 3, we compared the differential mRNA profiles when DEX was added in sandwich culture medium to those in absence of DEX to show, which genes encoding membrane transporters were directly affected by DEX treatment. During the first three days of culture, several kinds of modulations were observed due to the re-adaptation phase of the hepatocytes to their new environment. Interestingly, we showed that DEX modulated the mRNA level of genes encoding canalicular transporters such as mdr1b, mdr2, mrp2 but also some genes encoding membrane transporters which are located at basolateral membrane such as mrp3 and oatp2.

For the genes encoding basolateral transporters, a clear concentration-dependent modulation by DEX was observed for gene encoding mrp3 and for oatp2 on the second phase of the V-shaped GETP (Fig. 1). Based on the biliary excretion of taurocholic acid in sandwich culture, it has been demonstrated that hepatocytes partially restores bile acid uptake properties in this configuration [55]. Our results at mRNA levels showed no effect of DEX on the irreversible loss of *ntcp* expression over time. Assuming the same regulation mechanism in CPC and in SPC, a strong decrease in uptake functionality will also be expected in SPC for bile acids. Given that bile acids are also transported by oatp transporters into cells [56], oatp2 may act as a ''backup system'' which can be switched on in the case of down-regulation of the gene encoding ntcp. Studies are ongoing to show if this up-regulation at mRNA level is translated at functional level. An other effect of DEX was the net stabilization of mrp3 mRNA levels. As observed in CPC and in SPC in absence of DEX, the mRNA levels of *mrp3* was highly up-regulated over time. By adding DEX at 1 μM, *mrp3* mRNA levels were not modulated over time and close to baseline levels. The *mrp3* stabilization can thus be linked to the *mrp2* up-regulation demonstrating that *mrp3* and *mrp2* were interrelated and inversely regulated. As mrp2 was functional in SPC, this provide evidence that hepatocytes maintained in SPC in presence of DEX does not show the typical cholestatic state commonly observed in CPC.

For genes encoding canalicular transporters, our data showed an up-regulation of *mdr2* and *mrp2* mRNA levels by DEX as it was previously reported for *mrp2 in vitro* [53,57], and *in vivo* at the protein level [35]. Such an increase in *mrp2* and *mdr2* transcripts occurred after 20 hr exposure to the DEX and was directly associated with the formation of bile canalicular networks. It has been shown by Western blot analysis that this up-regulation was associated with elevated levels of mrp2 in cultured hepatocytes exposed to DEX [53]. Three mdr genes (*mdr1a*, *mdr1b* and *mdr2*) in rodents were shown to be implicated in drug resistance. GeneChip® data indicate that the three different genes encoding the P-glycoproteins were differently modulated by DEX. The gene encoding mdr2 was up-regulated in presence of DEX as shown earlier [15,57] whereas the *mdr1a* gene was not affected by DEX treatment. However, we observed that the *mdr1b* gene expression upon establishment of hepatocytes in SPC in presence of DEX was maintained to baseline level and was not induced to high levels as shown in CPC and in previous studies [49,54]. Consequently it appears that the *mdr* genes modulated by DEX involved mostly *mdr1b* and *mdr2* and not *mdr1a*. This modulation of P-gp levels in response to DEX treatment could rather be part of an early and marked alteration of differentiation status in untreated or exposed cultures to a low DEX concentration than a specific action of DEX on *mdr1* gene. Nevertheless other factors not directly related to the differentiation status, but which

can be linked to a cellular stress response, may also be involved in P-gp induction in cultured rat hepatocytes. This is based on the fact that addition of compounds, which favor maintenance of differentiated functions, did not completely abolish the P-gp increase in cultured rat hepatocytes. Therefore, the question is to be answered whether DEX regulates directly or indirectly the *mdr* genes knowing that no clear concentration dependence has been established so far for the induction and the stabilization of *mdr2* and *mdr1b*, respectively. Recent *in vivo* studies with mdr1 knockout mice provided a revealing picture of mdr1 type P-gp as an upstream regulator of hepatic *P450 3A* expression [60]. The results obtained from our studies in CPC showed a critical time dependent decrease of *CYP3A* mRNA level (data not shown) and an increased *mdr1b* expression. In contrast, in SPC, the gene encoding CYP3A1 was up-regulated by DEX and interestingly mdr1b was not modulated and maintained to the liver expression.[2] These data suggests that *mdr1b* and *CYP3A1* may be inversely regulated in cultured rat hepatocytes as it was demonstrated in adrenals [32].

Recent advances in the molecular biology of nuclear receptors have revealed that many genes are regulated by ligands which are involved in multiple hepatic physiological processes, including retinoids (RXR), oxysterols (LXR), bile acids (FXR), fatty acids (PPAR), and a variety of drugs and toxins. The pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) are able to act as sensors for lipophilic xenobiotics, including therapeutic drugs. These receptors regulate enzymes and transporters involved in drug metabolism and disposition in an adaptive fashion [58]. As example, several studies demonstrated that DEX modulates the *CYP3A* expression by the synergistic activation of the glucocorticoid receptor (GR) and the PXR [28,29,31,59]. As described for the C*YP3A23*, the regulation pathway can be divided in two major steps [61]. First, submicromolar concentrations of glucocorticoids increase cellular PXR and RXRα levels *via* a GR mediated mechanism. This leads to increased occupancy of the DEXRE-2 by PXR-RXRα and a low level transcriptional activation. Second, PXR is activated by DEX at higher concentrations resulting in further induction. Based on the point that hydrocortisone was present in medium from both culture model at 100 nM and that DEX was added as supplement in the medium used for sandwich culture, we can expect similar regulation pathways for some of these genes. Extensive literature data emerge actually concerning the involvement of nuclear receptors such as PXR in *CYP3A* and *mdr1* gene regulation. Recently, Kast *et al*. reported that *mrp2* is also a targeted PXR gene [62]. In the same way, Guo *et al*. has shown that the levels of rat *oatp2* mRNA and protein were increased by treatment with pregnenolone-16alpha-carbonitrile in both adult and in newborn rats. He indicates that the mechanism by which PCN induces rat *oatp2* gene is via interaction with PXR [63] and identifies rat *oatp2* as a direct PXR-targeted gene. The integration of the actions of these receptors in the metabolically active hepatocyte is proving to be complicated, with several of the key transporter genes already known to be regulated by multiple nuclear receptors. As example, *mrp2* has been shown to be regulated by at least four nuclear receptors, all acting within a very limited region of the promoter (RXR, FXR, RAR, CAR, PXR), while the *ntcp* promoter is regulated by at least four members (RXR, RAR, FXR and SHP), all *via* a single element [62]. The results presented in this study (up regulation of *CYP3A*, *mrp2* and *oatp2* by DEX, no effect of DEX on *ntcp* expression) combined with these findings further supports the hypothesis that DEX affects a network of genes that is involved in either metabolism or transport of drugs, steroids, and bile acids. Based on the fact that *ntcp* was not modulated by DEX, we may hypothesized that DEX, probably *via* PXR activation, induces transcription of the *oatp2* gene, which results in the uptake of organic anions and bile salts across the basal membrane. DEX *via* similar mechanism, also mediates the transcriptional induction of hepatic CYP3A, which, in turn, metabolizes a large number of endogenous and exogenous compounds. And finally, DEX activates the expression of genes encoding canalicular transporters, which is involved in the subsequent transport of many of these compounds into the bile. This coordinate regulation results in a net clearance of compounds from the blood (oatp2), detoxification of these compounds within the hepatocyte (CYP3A), and efflux of these compounds into the bile (mrp2), whereupon they can ultimately be excreted into the intestine and from the body. It showed also that multiple processes are involved in regulation of these genes and potentially interlinked *via* activation of common nuclear receptors.

The need of combined specific molecular biological assays (gene reporter assays, knock out mice, adenovirus mediated transfection) are still necessary for elaboration of mechanistic approaches such as the exploration of the regulation of genes in terms of nuclear receptors activation knowing that their expression were below the quantification limit by using oligonucleotide microarrays. The combination of such wide scale characterization of our *in vitro* models with more mechanistic approaches will further our understanding of the complex coordinate regulation of a biochemically set of genes that is important for xenobiotic disposition and elimination, whose expression is modulated in a coordinate manner so as to reduce the intracellular concentration of drugs, steroids, and bile acids.

In summary, the results suggest that neither isolation procedure nor the ECM in absence of DEX have a critical impact on gene expression of membrane transporters in hepatocytes. We demonstrated that hepatocytes even overlay by an ECM present a sort of cholestatic state with down-regulation at mRNA level of all basolateral trans-

[2] Luttringer *et al*. Transcriptomic and functional characterization of metabolizing enzymes in sandwich culture of rat hepatocytes, in preparation.

porters, up-regulation of mrp3 and mdr1b and down-regulation of mdr2. These findings provide further evidence that hepatocytes maintained in sandwich configuration in combination with a DEX-supplemented incubation medium in these fixed conditions re-established morphology close to that found *in vivo*, do not show cholestatic state and put in place compensation or backup systems to re-organize their detoxification. Taken together, these findings demonstrate that the hepatocytes in sandwich culture can be potentially used for morphogenesis and mechanistic transport studies but their use for quantitative prediction of transport needs to be further elucidated.

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