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Cellular Localization of Hepatic Cytochrome 1B1 Expression and Its Regulation by Aromatic Hydrocarbons and Inflammatory Cytokines

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ABSTRACT. Cytochrome P450 1B1 (CYP1B1) is an activator of several xenobiotics and is induced in the liver upon experimental exposure to aromatic hydrocarbons. Since its cellular localization and regulation are incompletely clarified, *Cyp1B1* expression and inducibility by 9,10-dimethyl-1,2-benzanthracene (DMBA) and inflammatory cytokines were investigated in different rat liver cell populations *in vitro* and in the liver during hepatocellular injury. Expression of *Cyp1B1* was studied by Northern blot analysis in hepatic stellate cells (HSCs), myofibroblasts (MFs), Kupffer cells (KCs), and hepatocytes at various time points of primary cultures and in acutely damaged rat liver (carbon tetrachloride model). Enzyme inducibility was assessed by incubation of cells with DMBA as well as, in the case of HSCs, with tumor necrosis factor-α (TNF-α) and transforming growth factorβ1 (TGFβ1). *Cyp1B1* messengers were expressed at high levels by HSCs and MFs, whereas constitutive expression was not detectable in KCs or in hepatocytes. *Cyp1B1*-specific mRNA were expressed at highest levels in HSCs at an early stage of activation (2 days after plating) and were diminished upon further activation. DMBA strongly enhanced *Cyp1B1* gene expression in HSCs, MFs, and in hepatocytes at day 3 of primary cultures, but not in hepatocytes at day 1, or in KCs. The inflammatory cytokine TNF-α enhanced the *Cyp1B1* gene expression in HSCs, either when administered alone or in addition to DMBA, while TGFβ1 did not affect *Cyp1B1* expression, even after DMBA induction. We conclude that HSCs and MFs seem to be the major cellular sources of hepatic *Cyp1B1* expression and that the constitutive expression of the *Cyp1B1* gene and the responsiveness to DMBA stimulation differ between mesenchymal and parenchymal liver cells, indicating a cell-specific regulation of *Cyp1B1* gene expression. Interestingly, TNF-α is a potent stimulator of the *Cyp1B1* gene in HSCs and acts in concert with DMBA. BIOCHEM PHARMACOL **58**;1:157–165, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. hepatic stellate cells; cytochrome P450 1B1; cytochrome P450 1A1; aryl hydrocarbon receptor; dimethylbenzanthracene; tumor necrosis factor-alpha

The recently identified Cyp1B1§ belongs to the P450 1 family [1–5], which also includes Cyp1A1 and Cyp1A2. The Cyp1 enzymes are active in the metabolism of several xenobiotics [6], such as PAH and heterocyclic amines, some of which have carcinogenic potential. The physiologic function of Cyp1B1 has been proposed to be that of a steroid hydroxylase [7]. The regulatory mechanisms of Cyp1 enzyme expression are still incompletely clarified. The mechanism of action of PAHs, the most potent inducers of *Cyp1* genes, includes several steps. They start by binding to the AHR, a cytosolic transcription factor, which is then translocated to the nucleus [8] and end up, after intermediate steps, acting on the *Cyp1* gene promoters [8, 9]. In addition, an AHR-independent pathway has been described for Cyp1B1 [10]. In addition to exogenous agents, endogenous substances, such as cytokines, affect the expression of *Cyp1* genes. In particular, cytokines have been found to have an inhibitor effect on the *Cyp1* genes studied to date [11, 12]. The expression of *Cyp1B1*-specific mRNA transcripts has been assessed in several organs, both in humans and rodents. In rats, a high constitutive expression was found in adrenals by Northern blot hybridization [5]. By use of the same technique, *Cyp1B1* mRNA transcripts were not detectable in any other rat organ, except for a minor expression in the testes [5]. In particular, *Cyp1B1*-specific

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§ *Abbreviations:* AHR, aryl hydrocarbon receptor; CCl_4, carbon tetrachloride; Cyp1A1, cytochrome P450 1A1; Cyp1B1, cytochrome P450 1B1; DMBA, 9,10-dimethyl-1,2-benzanthracene; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSC, hepatic stellate cell; KC, Kupffer cell; MF, liver myofibroblast; PAH, polycyclic aromatic hydrocarbon; TGFβ1, transforming growth factor β1; TNF-α, tumor necrosis factor-α and RT-PCR, reverse transcriptase-polymerase chain reaction.

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mRNA is undetectable by Northern blot in human [3, 13], rat [5, 14] and mouse [4] liver. However, a marked induction of the hepatic *Cyp1B1* gene occurs after experimental exposure to aromatic hydrocarbons in rodents [4, 5].

Very few studies on the cellular origin of hepatic *Cyp1B1* expression have been carried out thus far. By applying random priming PCR, we found *Cyp1B1* to be constitutively present in cultured HSCs and to be differentially expressed during the course of their *in vitro* activation. In healthy livers, HSCs are star-shaped cells mainly deputed to the metabolism of retinoids/vitamin A, in relation to which they contain large cytoplasmic deposits of lipids. At this stage, HSCs do not show any staining positivity for smooth muscle α-actin and can be referred to as "quiescent" HSCs [15]. When HSCs are set in primary culture, they gradually assume characteristics common to smooth muscle cells and myofibroblasts in a process called "activation" [15]. Interestingly, this *in vitro* "activation" process strongly resembles the morphological and functional changes observed in HSCs *in vivo* during liver fibrogenesis, and HSC primary cultures are thus a widely used *in vitro* model to gain insights into the role of these cells *in vivo*, in particular during hepatic fibrogenesis [15–18]. AHR knockout mice develop small livers and pronounced fibrosis of the portal tracts, whereas several other organs do not show any obvious histological abnormalities [19]. In the same AHR knockout animal model, a derangement of retinoid homeostasis, in which HSCs of healthy livers are profoundly involved, is also present, consisting in an excess of liver retinoid accumulation and diminished retinoic acid metabolism [20]. However, the enzymes connected with the AHR knockout phenotype and responsible for the alteration of retinoid metabolism have not yet been identified [20]. The aim of the present study was to investigate the expression and inducibility of the *Cyp1B1* gene in cultured HSCs with respect to other rat liver cell types and during the administration to HSC of cytokines involved in hepatic tissue repair and/or of a polycyclic hydrocarbon.

MATERIAL AND METHODS

Reagents

Chemicals were obtained from the following sources: Dulbecco's modification of Eagle's medium, M199 medium, and fetal bovine serum from Flow Laboratories, pronase E from Merck, collagenase of *Clostridium histolyticum* (used for hepatocyte isolations), random prime labeling kit and dNTPs from Boehringer, insulin S from Hoechst. Nycodenz was from Nyegaard. Collagenase type I, collagen type I (from rat tail), TGFβ1 prepared from human platelets, DMBA, and dexamethasone were from Sigma. Human recombinant TNF-α, exhibiting cross-reactivity with the rat system, was delivered by Genzyme. ^{35}S-dATP, ^{32}P-labeled dCTP, nick translation kit, and Hybond N membranes were obtained from Amersham Buchler. Reagents for random arbitrarily primed (RAP)-PCR were from Stratagene, and Moloney's murine leukemia virus reverse transcriptase and first strand synthesis buffer were purchased from GIBCO BRL. The TA cloning kit was from Invitrogen. The DNA sequencing kit was from Perkin Elmer.

Cell Cultures

Female Wistar rats provided by Charles River were maintained under 12-hr light/dark cycles with food and water *ad lib*. All animals received human care in compliance with institution and National Institutes of Health guidelines.

ISOLATION OF LIVER CELLS. Rat HSCs, liver KCs, and hepatocytes were isolated and purified according to standard protocols as described previously [17, 21–23]. Rat MFs were obtained by outgrowth of primary non-parenchymal liver cell cultures. Briefly, the liver was digested enzymatically with pronase and collagenase, and non-parenchymal liver cells were separated by a Nycodenz density gradient and further purified by centrifugal elutriation according to Knook *et al.* [24] and De Leeuw *et al.* [25]. Using a JE-6B elutriation rotor in a J2-21 centrifuge (Beckman Instruments) at 2,500 rpm, a cell fraction enriched with MF was collected at a flow rate of 23 mL/min. Specimens of whole rat organs (liver, spleen, kidney, heart, and brain) were also collected, snap frozen in liquid nitrogen, and kept at −80° until homogenization.

CELL CULTURE CONDITIONS. Plating of HSCs, KCs, and hepatocytes, and preparation and changing of the culture media were carried out as described earlier [17, 22, 26, 27]. Cells of the MF-enriched fraction were plated onto 24-well Falcon plates (Becton Dickinson) at a density of 5×10^5 cells per well in 1 mL culture medium. The medium was the same as for the HSCs and was changed twice a week. At the time of confluence, which was usually reached within 7–10 days, MFs were released from the culture plates by trypsinization and were replated at a 1:4 split ratio. MFs were again passaged at confluence under the same experimental conditions. MFs were subcultured for several passages, the experiments shown in this study being performed with MFs at passage 2 and 4. The purity of fresh cell preparations and of cell cultures was tested as outlined earlier and showed identical results to those previously described [17, 22, 26]. Using smooth muscle alpha actin (SMA) immunoreactivity as an activation parameter [15], HSCs were fully activated (100% SMA-positive) at day 7 of primary cultures. HSCs cultured for 2 days were mainly SMA-negative (<5% SMA-positive cells) and were classified as resting HSCs or HSCs at an early stage of activation.

Random Arbitrarily Primed (RAP)-PCR, Cloning, and Sequencing

Cyp1B1 was initially detected by RAP-PCR (also designated as differential mRNA display) through its down-regulation during spontaneous activation of HSCs from day 2 to day 7 after plating in primary cultures. For first strand

cDNA synthesis, 1 μg total RNA prepared from HSC at days 2 and 7 after plating was reverse transcribed using 200 U Moloney's murine leukemia virus reverse transcriptase and a single 18-base arbitrary primer (primer sequence: AAT CTA GAG CTC CTC CTC). Out of the total reverse transcriptase reaction of 20 μL, 1 μL was used as template DNA for PCR. PCR was performed for a single round under low stringency conditions (5 min each at 95°, 36°, and 72°) and 40 cycles under high stringency conditions (1 min at 95°, 2 min at 50°, and 2 min at 72°) with the above-mentioned 18-base primer and ^{35}S-dATP. Final elongation time was 10 min at 72°. PCR products were analyzed by electrophoresis in a 4% acrylamide/7 M urea sequencing gel followed by autoradiography. Differentially expressed PCR products were excised from the gel, dissolved in TE, and reamplified using 40 cycles under high stringency conditions (1 min at 95°, 2 min at 50°, and 2 min at 72°) with the above-mentioned 18-base arbitrary primer. Out of the total PCR reaction of 50 μL, 10 μL was analyzed in a 1% agarose gel. PCR products were cloned into the pCR^{TM}2.1 cloning vector using the TA cloning kit, and several clones were sequenced using the DNA sequencing kit from Perkin Elmer. Sequence comparison was performed by the FASTA, BestFit, or BlastN alignment programs using standard parameters [28, 29]. One PCR product, with a length of 1076 bp and an apparent down-regulation in fully "activated" HSCs in comparison to HSCs at an early stage of activation as seen at autoradiography of the urea gel, corresponded to the rat *Cyp1B1* sequence, published mRNA positions 2573–3648 (AC U09540) [5].

Cell Stimulation

STIMULATION WITH DMBA. DMBA was dissolved in absolute ethanol. Three stocks were prepared and used for all experiments to produce final concentrations of 0.2, 10, and 500 nM after addition to the medium. The final amount of ethanol in the medium was 1 μl/mL. Stimulations were performed at days 2 and 7 after plating for HSCs, at days 1 and 3 in case of KCs and hepatocytes, and at the 2nd and 4th passage for MFs. Cells were washed three times with Gey's balanced salt solution and incubated for 20 hr before RNA extraction. The incubation medium was serum-reduced (0.3% fetal bovine serum) for HSCs, MFs, and KCs, whereas Dulbecco's modified Eagle's medium with 0.05% insulin plus 0.2% BSA was used in the case of hepatocytes. The stimulation was carried out by use of the incubation medium alone or with the addition of ethanol at 1 μl/mL—the same concentration as that used to vehicle DMBA—or with ethanol plus DMBA. Experiments with hepatocytes were performed both without and with the addition of 10^{-7} mol dexamethasone to the incubation medium.

STIMULATION OF HSCS WITH TNF-α AND TGFβ1. HSCs were washed three times with Gey's balanced salt solution at day 2 and day 7 after plating and incubated for 20 hr in serum-reduced (0.3% fetal bovine serum) culture medium alone, with TNF-α (100 U/mL) and/or TGFβ1 (1 ng/mL). The concentrations of cytokines used had been previously shown to have no cytotoxic effects [18, 27]. HSCs at day 4 (intermediate stage of "activation") were also stimulated with DMBA alone (10 and 500 nM) or with DMBA plus TNF-α (100 U/mL) or TGFβ1 (1 ng/mL) to evaluate the effect of cytokines on Cyp1B1 inducibility by DMBA.

INDUCTION OF ACUTE LIVER DAMAGE. Rats were given CCl_4/maize oil solution (50% w/w) by oral administration as previously described [17, 22]. CCl_4 dosage was 75 μL/100 g body weight. Control animals were treated with maize oil only. Four animals in each group were killed 3, 6, 9, 12, 24, 48, 72, and 96 hr after a single high-dose CCl_4 administration. The liver was perfused with saline solution (0.9% NaCl) and snap-frozen in liquid nitrogen.

RNA Extraction, Northern Blot Analysis, and RT-PCR

RNA EXTRACTION AND NORTHERN BLOT ANALYSIS. Total RNA of HSCs, KCs, hepatocytes, and MFs was extracted from cell cultures at the different time points and, in the case of HSCs, KCs and hepatocytes, also from freshly isolated cells. Liver cells were lysed with guanidium isothiocyanate and total RNA was extracted by ultracentrifugation on $CsCl_2$ as described elsewhere [17, 22, 30]. Whole rat tissues were lysed in guanidium isothiocyanate by means of a mechanical homogenizer and total RNA was extracted as for cell cultures. Total RNA was resolved by agarose gel electrophoresis, transferred to nylon membranes, and hybridized with specific cDNA probes. Total RNA from cellular cultures (5 μg) or extracted from tissues (10 μg) was loaded on each lane. Hybridization was performed for 2 hr at 68° using the QuickHyb Kit (Stratagene). Posthybridization washes were performed twice, for 10 min each time, at room temperature and once for 3–15 minutes at 60° in 2X standard saline citrate containing 0.1% SDS. Nylon filters were washed, dried, and exposed to x-ray films at −80°. After adequate exposure, the filters were washed in 1 M hot Tris–EDTA buffer and then rehybridized. Hybridizations were performed against *Cyp1B1*, *Cyp1A1*, *AHR* and subsequently GAPDH or 28S RNA. Northern blot results shown in Figures 1–5 are representative of several independent experiments.

cDNA Probes

CYP1B1. The 1076 bp long cDNA fragment, which was generated by the differential display method and corresponded to the published sequence of rat *Cyp1B1* position 2573–3648 (AC U09540) [5], was used as probe. Rat *Cyp1B1*-specific messengers were detected at 5.2 Kb by Northern blot hybridization. In addition to prominent transcripts at 5.2 Kb, *Cyp1B1*-specific messengers, signaling

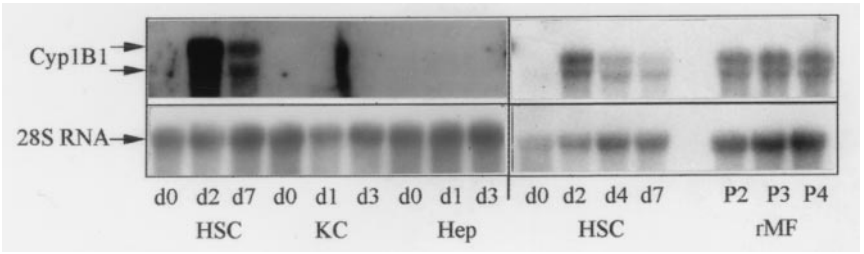

FIG. 1. *Cyp1B1*-specific transcript expression in freshly isolated and primary cultures of liver cells. Total RNA was extracted from freshly isolated (d0) and cultured cells (d = day after plating, P = passage in primary culture). rMF, rat myofibroblasts; Hep, hepatocytes. 28S RNA was used to assess the RNA load of the lanes.

slightly below 28S RNA, were present in accordance with the literature [5].

Cyp1A1. A plasmid containing the sequence for *Cyp1A1* (pSV4501A1) was a kind gift of Dr. Karen I. Hirsch-Ernst (Institute of Pharmacology and Toxicology, University of Göttingen, Germany). It hybridizes to *Cyp1A1* messengers of 2.9 kb and has been described in detail elsewhere [31, 32].

AHR. A 259 bp long cDNA generated by RT-PCR using HSC-derived samples was employed for Northern blot analysis. Primers for the RT-PCR were designed using the "prime" program from the Wisconsin package of the genetics computer group [29] and were as follows: forward primer, 5′-TGA CGG ATG AAG AAG GAC-3′, reverse primer, 5′-GGA GGA CAC AGA TAG ATG G-3′, corresponding to the published nucleotide sequence of the rat mRNA for *AHR*, positions 1151–1409 and 1409–1391 (AC U09000) [33]. The PCR product was cloned into the pCR^{TM}2.1 cloning vector using the TA cloning kit and sequenced.

To validate quantitative Northern blot results, clones carrying the rat GAPDH cDNA [34] or an oligonucleotide directed against 28S RNA [35] were used. cDNA probes were ^{32}P-labeled by random priming (Cyp1B1, AHR, and GAPDH) or nick translation (Cyp1A1 and 28S RNA).

RT-PCR

Specific primers for *Cyp1B1* were designed as described for the AHR. The primers were synthesized (MWG Biotech) as follows: forward primer 5′-ACC AAC CCA ACT TAC CAT AC-3′ and reverse primer 5′-TCT TAG ACA ACT CCT CGC C-3′, corresponding to positions 1492–1511 and 1803–1785 (AC U09540) [5] and encompassing a PCR product of 312 bp. The PCR was performed for 40 cycles with cycle times of 1 min at 95°, 1.5 min at 55° (*Cyp1B1*) or 60° (*AHR*), and 2 min at 72°. Final elongation time was 10 min at 72°. Out of the total PCR reaction of 50 μL, 10 μL was analyzed in a 2% agarose gel. To validate RT-PCR results, PCRs using GAPDH-specific primers were performed as described [30]. The RT-PCRs were repeated on individual cell preparations of at least three independent cell cultures.

RESULTS

Initially, we detected *Cyp1B1* mRNA via random arbitrarily primed PCR in cultured HSCs, due to its down-regulation during the course of spontaneous *in vitro* activation of these cells. This was confirmed by Northern blot hybridization (Fig. 1), as *Cyp1B1* mRNA levels were down-regulated from day 2 to day 7 of primary cultures. Interestingly, *Cyp1B1* messengers were absent in Northern blot analysis of freshly isolated HSCs. To study the relative contribution of HSCs with respect to other liver cell types, *Cyp1B1* expression was also analyzed by Northern blot in hepatocytes, MFs, and KCs. As in HSCs, no *Cyp1B1* expression was apparent in freshly isolated hepatocytes and KCs (Fig. 1). While *Cyp1B1*-specific transcripts spontaneously appeared in primary cultures of HSCs, *Cyp1B1*-coding mRNAs were not detectable by Northern blot hybridization in total RNA prepared from cultivated hepatocytes and KCs (Fig. 1). In MFs, *Cyp1B1* mRNA expression was similar to that of HSCs at day 2 and did not show significant changes between passages 2 and 4 (Fig. 1).

Effects of DMBA on Cyp1B1, Cyp1A1, *and* AHR *Expression in HSCs and MFs*

Cyp1B1 expression was enhanced both in HSCs and in MFs by DMBA at concentrations ranging from 10 to 500 nM (Fig. 2). The enhancement was dose-dependent and was present at all time points of culture. *Cyp1A1* messengers, studied as control for the effect of aromatic hydrocarbons, were not detectable by Northern blot analysis in untreated HSCs and MFs. However, they were induced in MFs by 500 nM DMBA. The same DMBA concentration induced a similar expression of *Cyp1A1* in HSCs at day 2. In contrast, in fully "activated" (day 7) HSCs, *Cyp1A1* was only barely detectable following prolonged autoradiographic exposures under stimulation with 500 nM DMBA (Fig. 2). *Cyp1A1* hybridized as a single band to mRNA of 2.9 Kb; no cross hybridization with *Cyp1A2*, whose transcripts are located at 2.1 Kb, was apparent. Rat *AHR*-specific transcripts were detected above the 28S RNA in a size range similar to human *AHR* (6.6 Kb) (Fig. 2). They were constitutively expressed in unstimulated HSCs and MFs. In untreated MFs, no apparent difference was present between passage 2 and 4, whereas in untreated HSCs a down-regulation occurred during *in vitro* activation from day 2 to 7. Stimulation with DMBA dose dependently enhanced the expres-

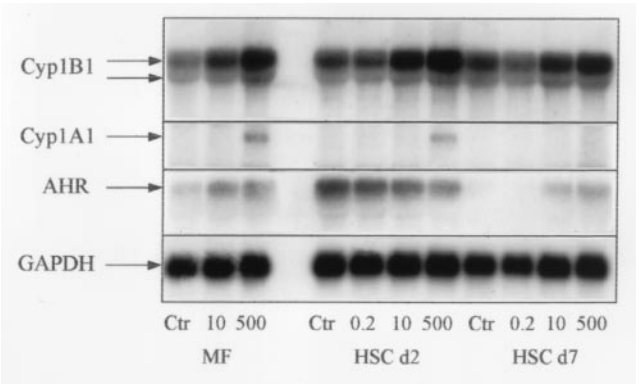

FIG. 2. *Cyp1B1*, *Cyp1A1*, and *AHR* expression in HSC and MF under stimulation with DMBA. HSC d2, hepatic stellate cells at day 2 of primary culture, also designated as HSC "at an early stage of activation"; HSC d7, fully "activated" HSC, at day 7 of primary culture; and Ctr, controls (ethanol 0.1 μL/mL medium). Stimulation of cells by DMBA was performed at three different DMBA concentrations: 0.2, 10, and 500 nM. The figure represents one of three similar experiments. GAPDH RNA was used to assess the RNA load of the lanes.

sion of *AHR* transcripts both in MFs and in "activated" HSCs (day 7). On the contrary, in HSCs at day 2 a dose-dependent down-regulation of *AHR* mRNA expression occurred under stimulation with DMBA, apparently in a dose-related manner.

Expression of Cyp1B1, Cyp1A1, *and* AHR *in Hepatocytes and KCs and Effects of DMBA*

In untreated hepatocytes (Fig. 3) and KCs (Fig. 1), Cyp1B1-specific transcripts were not detectable by Northern blot hybridization at any time point of culture. In hepatocytes at day 1 of primary culture, *Cyp1B1* mRNA did not become evident upon Northern blot analysis after stimulation with DMBA, at any concentration tested. By contrast, at day 3 of culture, 500 nM DMBA induced the expression of the *Cyp1B1* gene. No difference was observed in hepatocytes stimulated in the presence or absence of dexamethasone. In KCs an induction of *Cyp1B1* gene was not visualized by Northern blot either at day 1 or at day 3 of culture. *Cyp1A1*-specific transcripts were undetectable by Northern blot hybridization in hepatocytes at day 1 of culture in the absence of dexamethasone (Fig. 3) or in KCs (data not shown). On the contrary, *Cyp1A1* expression was evident in hepatocytes at day 1 in the presence of dexamethasone and in hepatocytes at day 3 irrespective of dexamethasone stimulation (Fig. 3). Stimulation with 500 nM DMBA enhanced or induced the *Cyp1A1* gene in hepatocytes at all culture time points and under all conditions (Fig. 3). *AHR*-specific transcripts were highly expressed in hepatocytes both at day 1 and 3 of culture (Fig. 3), but were barely detectable in KCs (data not shown). Neither DMBA stimulation nor the presence of dexamethasone in the incubation medium of hepatocytes modified its expression.

Effects of TNF-α and TGFβ1 on Cyp1B1, Cyp1A1, *and* AHR *Expression in HSCs*

TNF-α enhanced *Cyp1B1* gene expression in HSCs. The stimulatory effect was evident both in HSCs at day 2 after plating and in fully "activated", myofibroblast-like HSCs (day 7 after plating) (Fig. 4). The spontaneous down-regulation of *Cyp1B1* transcript expression from day 2 to day 7 of culture was also evident in these experiments (Fig. 4). In addition, TNF-α acted additive to the stimulation of *Cyp1B1* expression induced by DMBA (Fig. 5). TGFβ1 had

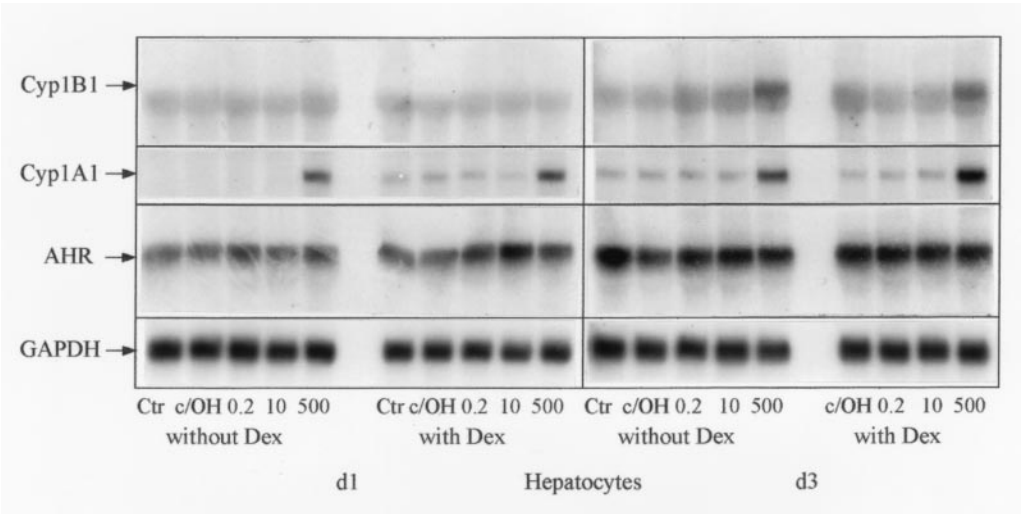

FIG. 3. *Cyp1B1*, *Cyp1A1*, and *AHR* expression in hepatocytes under stimulation with DMBA. Hepatocytes were studied at day 1 (left panel) and day 3 (right panel) of primary cultures. Ctr, controls without vehicle and c/OH, controls with vehicle (ethanol 0.1 μL/mL medium). Stimulation of cells in primary cultures by DMBA was performed at three different DMBA concentrations: 0.2, 10, and 500 nM. Stimulation with DMBA was performed in the presence or absence of dexamethasone (Dex, 10^{-7} M) in the stimulation medium to evaluate the two commonest conditions of hepatocyte cultivation generally used. The figure represents one of three similar experiments. GAPDH RNA was used to assess the RNA load of the lanes.

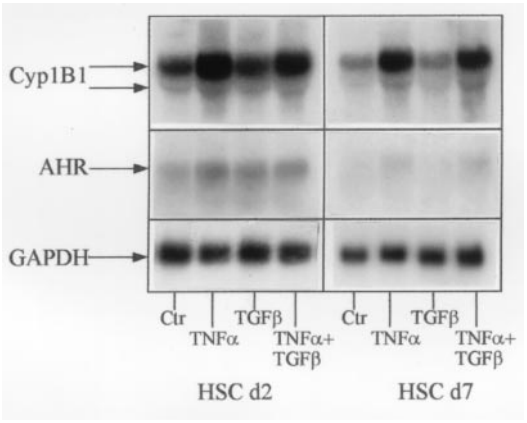

FIG. 4. *Cyp1B1* and *AHR* expression under stimulation with TNF-α and TGFβ1. HSC d2, hepatic stellate cells at day 2 of primary culture, also designated as HSC "at an early stage of activation"; HSC d7, fully "activated" HSC, at day 7 of primary culture; and Ctr, controls. Stimulation of cells in primary cultures was performed with TNF-α (100 U/mL), TGFβ1 (1 ng/mL), or both. GAPDH RNA was used to assess the RNA load of the lanes.

no substantial effect on *Cyp1B1* mRNA expression nor on its inducibility by TNF-α (Fig. 4) or DMBA (Fig. 5). *Cyp1A1* messengers were undetectable in untreated HSCs by Northern blot analysis both at day 2 and 7 of primary cultures. Neither TNF-α nor TGFβ1 (Fig. 5) nor the two together were able to induce the expression of *Cyp1A1* in HSCs at any time point of culture. Simultaneous stimulation with 500 nM DMBA and TNF-α revealed an inhibitory effect of this cytokine on *Cyp1A1* transcripts (Fig. 5), contrary to the effect observed on Cyp1B1. This effect of TNF-α on *Cyp1A1* expression could not be assessed in HSCs not stimulated with DMBA or with DMBA at lower concentrations than 500 nM, since *Cyp1A1*-specific transcripts were not detectable by Northern blot analysis under these conditions, regardless of TNF-α stimulation. TGFβ1 did not have any major effect on *Cyp1A1* expression, similar to what was seen for *Cyp1B1* in HSCs (Fig. 5). The effects of TNF-α and TGFβ1 on constitutive *AHR* gene expression were qualitatively similar to those on the

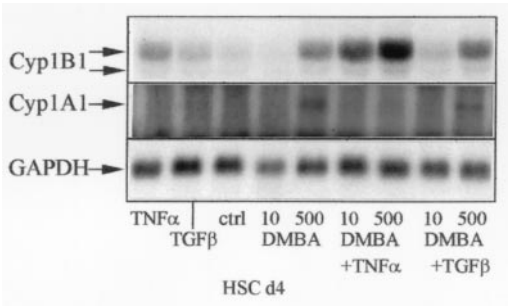

FIG. 5. *Cyp1B1* and *Cyp1A1* expression under stimulation with TNF-α or TGFβ1 or together with DMBA in HSC at day 4 of primary culture. Stimulation of HSCs was performed with TNF-α (100 U/mL) or TGFβ1 (1 ng/mL) alone or in addition to DMBA at concentrations of 10 and 500 nM. Ctrl, controls. GAPDH RNA was used to assess the RNA load of the lanes.

Cyp1B1 gene. TNF-α enhanced the *AHR* gene, both in the presence and absence of TGFβ1, which did not show any substantial effect (Fig. 4). Moreover, in these experiments a down-regulation of AHR-specific transcript expression was apparent during the course of HSC activation (Fig. 4).

Expression of Cyp1B1, Cyp1A1, *and* AHR *in Total Liver RNA*

From the earlier experiments, it became evident that HSCs and MFs express significant amounts of *Cyp1B1*-specific messengers *in vitro*. On a μg cellular RNA basis, the highest *Cyp1B1* expression levels were detected in early cultured HSC. Freshly isolated HSCs, as well as KCs and hepatocytes, which are believed to correspond in part to the cells present in the liver, displayed no *Cyp1B1* expression (Fig. 1), suggesting that *Cyp1B1* in normal liver is low. Indeed, as assessed by Northern blot analysis of total RNA prepared from normal liver tissue, *Cyp1B1*-specific transcripts were not detectable (data not shown). We also tested whether hepatic *Cyp1B1* expression could be modulated by acute liver damage induced by CCl_4 treatment. In this model, hepatocellular injury is accompanied by a rapid increase in TNF-α expression in the liver [36], and this cytokine appears to enhance the *Cyp1B1*-specific message in HSCs *in vitro*. However, no signal from *Cyp1B1* transcripts became evident as assessed by Northern blot analysis of total RNA from the whole organ (data not shown). In addition, *Cyp1B1* expression was not apparent in total RNA prepared from other organs such as spleen, heart, brain, and kidney. Furthermore, neither *Cyp1A1* nor *AHR* genes were detectable in the total RNA from these various tissues by Northern blot hybridization. However, as assessed by RT-PCR, both *Cyp1B1* and *AHR* mRNAs were detectable in the liver and in the other tissues tested, illustrating that, in accordance with the data obtained from freshly isolated cells, constitutive *Cyp1B1* expression is low and therefore undetectable by Northern blot techniques.

DISCUSSION

The present study reports the expression and regulation of *Cyp1B1* mRNA in four different liver cell populations. Our results show that *Cyp1B1* mRNA is constitutively expressed at high levels in primary cultures of liver cells of the fibroblastic lineage, namely MFs and HSCs. In these cells, DMBA enhanced *Cyp1B1* gene expression in a dose-related manner. In addition, TNF-α acted additive to the stimulation of *Cyp1B1* expression induced by DMBA. An enhancing effect of TNF-α was also present in the case of *AHR*-specific transcripts, which might be the underlying mechanism for the enhancement of TNF-α-mediated *Cyp1B1* expression.

The predominant expression of *Cyp1B1* in mesenchymal liver cells is in agreement with previous works analyzing other organs: *Cyp1B1*-specific transcripts were found to be differently expressed in mammary fibroblasts and parenchy-

mal cells in culture [37]. In detail, in primary cultures of cells of the rat mammary gland—where DMBA is one of the most potent carcinogenic substances—*Cyp1B1* is expressed at low constitutive levels and is substantially induced in fibroblasts, but not in epithelial cells. Conversely, constitutive *Cyp1A1* expression is absent, but can be induced, in rat mammary epithelial cells, whereas it is not expressed or inducible in stromal fibroblasts [37]. Our data indicated that *Cyp1A1* and *Cyp1B1* are also induced by aromatic hydrocarbons in a cell-specific pattern in the liver, as found previously in other organs [38–40] and that, in the liver, *Cyp1B1* is expressed preferentially in non-parenchymal cells. In addition to the different *Cyp1B1* expression in cells of the fibroblast lineage and parenchymal cells/macrophages, our data indicate that *Cyp1B1* expression also depends on the activation/differentiation profile of a cell. *Cyp1B1*-specific transcripts were not detectable in "quiescent" HSC (freshly isolated cells), but were expressed at very high levels in HSC at an "early stage of activation" (day 2 after plating). Thereafter, their expression decreased to lower levels in fully "activated" HSC. However, despite the changes in constitutive expression, the responsiveness to DMBA stimulation was preserved both in HSCs "at an early stage of activation" and in fully "activated" HSCs.

Induction of *Cyp1A1* mRNA was used as a control of the DMBA effect *in vitro*. Indeed, *Cyp1A1*-specific transcripts were inducible both in MFs and in HSCs. However, in this case as well, a peculiar behavior was observed in HSCs. HSCs at an "early stage of activation" (day 2 of primary culture) showed an induction of *Cyp1A1* gene expression following treatment with 500 nM DMBA, whereas this induction was not evident in fully "activated" HSCs (day 7) under the same experimental conditions (Fig. 2). These findings are in accordance with a previous work on murine keratinocytes, which reported that *Cyp1A1* and *Cyp1B1* expression is specifically regulated in relation to the differentiation status of the cells [41]. Furthermore, the different *Cyp1B1*–*Cyp1A1* pattern observed in MFs and in "activated"/myofibroblast-like HSCs could be regarded as additional evidence that these two cell populations of fibroblast lineage are not identical.

The expression of *Cyp1B1* seems to rely mainly upon the presence of the AHR [8, 42]. Interestingly, a marked down-regulation of *AHR* expression was observed during the course of activation of HSCs and paralleled the expression profile of *Cyp1B1* in the same cell type. However, even though the changes in *AHR* gene expression might provide an explanation for the decrease in *Cyp1B1*-specific messengers, the interaction between the two genes is more complex involving as it does postreceptor pathway factors [43]. *Cyp1B1* mRNA expression showed an enhancement in HSCs under stimulation with DMBA, both at day 2 and 7 of primary cultures. Furthermore, *AHR* gene expression was enhanced by DMBA at day 7 of culture, but was reduced under DMBA stimulation at day 2 in a dose-related manner. This latter finding appears to be new and peculiarly present in the liver only in HSC at an "early stage of activation". The AHR seems to be involved in important processes such as organ development and cell cycle regulation [38, 44]. It is tempting to hypothesize a role for the AHR in HSC activation as well. In fact, transgenic *AHR*-knockout mice show histological alterations specifically in the liver, developing small livers and fibrosis of the portal tracts [19]. In addition, they exhibit liver retinoid accumulation and reduced retinoic acid metabolism [20]. It is interesting to note that "activated" HSCs, which represent the main cell type responsible for liver fibrogenesis and are characterized by low vitamin A content, show a marked down-regulation of *AHR* mRNA expression. Apart from the AHR, a potential involvement of Cyp1B1 in the regulation of the extracellular matrix environment, which could be invoked by the present finding of *Cyp1B1* mRNA reduction during HSC activation, is suggested by the data from another organ [45]: an abnormally low expression of the Cyp1B1 enzyme in the trabecular meshwork cells of the eye, which regulate the extracellular matrix deposition of this tissue, causes primary congenital glaucoma [45]. However, focused experimental data on this topic are warranted before drawing any conclusion in the liver.

Cyp1B1 messengers were inducible solely by 500 nM DMBA in hepatocytes cultivated for 3 days. On the contrary, *Cyp1A1* mRNA, used as a control for the effects of aromatic hydrocarbons, was inducible in hepatocytes by DMBA both at day 1 and 3 after plating, in accordance to published data [31, 46]. Constitutive expression of *Cyp1A1*-specific transcript was absent in untreated hepatocytes at day 1. An expression was instead visible in the presence of dexamethasone in the medium. No major modification of *AHR* gene expression was evident in hepatocytes at the different time points, nor was this expression modified by stimulation with DMBA or by the presence of dexamethasone. These findings, taken together and compared to those of HSCs, MFs, and KCs, further confirm the cell-specific pattern and regulation of the enzymes of the cytochrome P450 1 family [37, 38, 40] and the contribution of postreceptor regulatory factors in this cell specificity [8, 9, 43, 46].

In addition to the known effects of aromatic hydrocarbons on *Cyp1B1* expression, we demonstrated that the inflammatory cytokine TNF-α induces *Cyp1B1* and *AHR* messengers in HSCs. An inhibition of cytochrome P450-related enzyme activities has been shown during infection and inflammation [47, 48] and has been attributed to increased levels of proinflammatory cytokines, such as TNF-α. Indeed, TNF-α down-regulated the DMBA-induced expression of *Cyp1A1* in hepatocytes [12] and we have shown a similar effect in HSCs here. However, we demonstrated that TNF-α has an opposite effect on *Cyp1B1*. This stimulatory effect of TNF-α is a new finding, since the previous known effects of various cytokines, such as TNF-α, TGFβ1, and interleukins on Cyp1 enzymes, appeared to be inhibitory [12]. Such divergent effects of the same cytokine on *Cyp1B1*- and *Cyp1A1*-specific messengers suggest a very important role of posttranscriptional

factors [9], as the induction of both enzymes by PAH is mediated by the AHR. TNF-α, which raises *Cyp1B1* expression in HSCs, is expressed at high levels at the onset of hepatic injury, mediating a variety of biological events, in particular hepatic inflammation. Accordingly, the CCl_4 model of hepatocellular injury is accompanied by a rapid increase in TNF-α expression in the liver [36]; TNF-α peaks at 9–12 hours and returns to baseline levels at 48 hr. However, no signal from *Cyp1B1* transcripts became evident after acute CCl_4 administration, as assessed by Northern blot analysis of total RNA from the whole organ (data not shown). Whether this finding is due to the relatively low amount of HSC in the liver or to an ineffectiveness of TNF-α on *Cyp1B1* expression *in vivo* awaits further experimental analysis.

In our experiments, TGFβ1 did not significantly modify the basal and DMBA-induced expression of Cyp enzymes or of AHR. Regulation of *AHR*, *Cyp1B1*, and *Cyp1A1* by TGFβ1 therefore appears to be slightly different in HSCs from was what found in a human lung tumoral cell line and in primary cultures of hepatocytes, in which TGFβ1 down-regulates both basal and aryl hydrocarbon-induced expression of these genes [11, 12]. Such differences may be interpreted as interspecies diversities or as a further confirmation of cell-specific and differentiation status-specific regulations.

Neither *Cyp1B1* nor *Cyp1A1* transcripts were visualized by Northern blot analysis of total RNA prepared from whole organs. This observation is consistent with previous studies analyzing rat [5] and mouse [1] tissues. In humans, on the contrary, *Cyp1B1* seems to be constitutively expressed in several extrahepatic organs [3], but not in the liver, indicating low [49], if any, *Cyp1B1* expression in this organ.

In conclusion, we have shown that *Cyp1B1*-specific transcripts are constitutively expressed in primary cultures of liver cell populations, particularly in HSCs and MFs, and that inducibility by aromatic hydrocarbons is differently regulated according both to the cell type and the differentiation status of the cells. Furthermore, *Cyp1B1* mRNA was shown to be inducible not only by aromatic hydrocarbons but also by the endogenous cytokine TNF-α.

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