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SHORT COMMUNICATION

No Significant Expression of *CYP2E1* in Rat Liver Stellate Cells

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ABSTRACT. The putative role of the ethanol-inducible cytochrome P450(CYP)2E1 in stimulating collagen synthesis by rat liver stellate cells was studied. Analysis of carefully isolated stellate cells revealed that their content of immunoreactive CYP2E1 protein and of CYP2E1 mRNA, as determined by reverse transcription-polymerase chain reaction (RT-PCR), was very low, i.e. only 0–4% of that in hepatocytes. We conclude that it is improbable that such low expression of CYP2E1 in stellate cells would have functional importance. BIOCHEM PHARMACOL **56**;8:1075–1078, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. CYP2E1 expression; rat liver; stellate cell isolation; collagen 1; fibrogenesis

Stellate cells, also called Ito cells, lipocytes, or fat-storing cells, play a crucial role in hepatic fibrogenesis. Following chronic ethanol feeding [1] or CCl_4 intoxication [2] these cells are transformed to transitional cells which produce substantial amounts of extracellular matrix proteins, most importantly type I collagen. The factors regulating the expression of collagen I gene *in vivo* are not fully understood. Studies with isolated stellate cells suggest that at least acetaldehyde (the first metabolite of ethanol) [3–5], transforming growth factor β1 [5], and free radical-initiated lipid peroxidation [6] are involved in stimulation of collagen I synthesis.

CYP2E1§ is an ethanol-inducible member of the P450 superfamily. This enzyme catalyzes numerous reactions, including the oxidation of ethanol to acetaldehyde. The enzyme has an unusually high capacity to generate free radicals [7], which, in turn, stimulate lipid peroxidation. The expression of *CYP2E1* is highest in hepatocytes, but is also expressed and induced in Kupffer cells [8, 9]. According to a recent report, rat stellate cells would also express CYP2E1 protein at a relatively high level: 21% (relative to protein) of that in hepatocytes [10]. At this level of expression, CYP2E1 could be of functional importance, mediating the local acetaldehyde formation and lipid peroxidation thought to stimulate hepatic fibrogenesis. In view of this, we decided to reinvestigate *CYP2E1* expression in stellate cells, both at the protein and mRNA level, by using immunoblotting and RT-PCR techniques.

MATERIALS AND METHODS

Stellate cells were isolated from 9-month-old male Wistar rats. Livers were perfused *in situ* under pentobarbital anesthesia (60 mg/kg i.p.), first with Ca^{2+} and Mg^{2+} free HBSS for 10 min and then with normal HBSS containing 0.1% pronase E and 0.5% collagenase (both from Sigma) for another 10 min. Livers were minced and incubated at 37° with constant shaking for 45 min in HBSS containing 0.02% pronase and 0.05% collagenase. Hepatocytes were removed by centrifuging at 50 *g* for 2 min, and the stellate cells were isolated using single-step Nycodenz (Sigma) density gradient centrifugation as described by Schäfer *et al.* [11] with minor modifications. To improve the recovery and purity of stellate cells, a third layer of 8% Nycodenz was added between 11.4% Nycodenz and GBSS layers. After centrifugation, the stellate cells were collected from the 8% Nycodenz-GBSS interphase. The cells were then washed 3 times (450 *g*, 7 min, +4°) and resuspended in a small volume of GBSS. Stellate cells were identified by their typical microscopical appearance with characteristic fat droplets. The contamination of the stellate cell preparation with much larger hepatocytes in each preparation was estimated by counting at least 700 cells under phase-contrast microscope. No significant contamination of Kupffer cells was observed. Separate hepatocyte preparations were obtained from the same rats as the stellate cells after the first centrifugation step. The viability of the hepatocytes was 70–80%.

The relative protein contents of stellate cell and hepatocyte preparations were analyzed by SDS-PAGE in 8–20% PhastGels® followed by silver staining with PhastGel®

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§ *Abbreviations:* CYP, cytochrome P450 gene; CYP, cytochrome P450 protein and mRNA; GBSS, Gey's Balanced Salt Solution; HBSS, Hank's Balanced Salt Solution; and RT-PCR, reverse transcription-polymerase chain reaction.

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Silver kit (Pharmacia LKB Biotechnology AB). The expression level of CYP2E1 protein was analyzed by immunoblotting, followed by videodensitometry quantitation of the filters, essentially as recently described [12]. Rabbit antiserum to rat CYP2E1 [13], kindly donated by Dr. Ingelman-Sundberg (Karolinska Institute, Stockholm, Sweden), and the reagents of the Western Light chemiluminescent detection system with alkaline phosphatase (Tropix, Inc.) were used for detection of CYP2E1 protein, except that the final step was a colorimetric detection with Amresco's (Solon) BCIP-NBT substrate. Band intensities of the samples were normalized to the intensity of a standard hepatocyte sample run on each gel.

The relative amounts of CYP2E1 transcripts were analyzed using a semiquantitative RT-PCR method as described with other primers elsewhere [12, 14]. To obtain enough RNA, stellate cell preparations with similar hepatocyte contaminations were pooled, and the total RNA was isolated using an RNeasy kit from Qiagen. The first strand of cDNA was produced with Promega's Reverse Transcription System from 1 μg of RNA in a 20-μL reaction volume, according to the manufacturer's instructions. Specific primers for CYP2E1 mRNA, 5′-GAGGCGCAATTCCTGGT GGAGGAGC-3′ (forward) and 5′-TCATAGTTTA AGGGATAACA-3′ (reverse), were used to amplify a 331 bp fragment from 0.3 to 5 μL of cDNA. A 100-μL reaction volume contained 2 U of Taq DNA polymerase, 1 × PCR buffer (both from Boehringer Mannheim), 50 pmol of both primers, 0.2 μM of each dNTP (Promega), and 3 mM $MgCl_2$. Twenty cycles (95° for 1 min, 55° for 1 min, and 72° for 1 min) of PCR were performed. The last elongation step was extended to 5 min. Relative quantitation of the PCR products was performed by anion exchange HPLC [15]. The linearity of amplification was validated in separate experiments by varying the amount of cDNA, the number of cycles, and the amount of RNA in cDNA synthesis. Samples to be compared were always run together. The amount of CYP2E1 transcripts in each sample was normalized to relative amplification of the same cDNA with β-actin primers, as recently described [16]. In addition to HPLC, the amplification products were analyzed by PAGE in 20% PhastGels® (Pharmacia) with a native buffer system followed by staining with PhastGel® Silver kit according to the manufacturer's instructions.

The results, i.e. the CYP2E1 immunoreactive protein or mRNA, are expressed as percentage of the CYP2E1/μg of protein or/β-actin mRNA in average hepatocyte preparations (N = 8 or 9) and plotted against the percentage of hepatocyte contamination. A linear regression with the 95% confidence interval is presented; Pearson correlation coefficient *r* and two-tailed *P* value calculated with GraphPad (San Diego) Prism™ software.

RESULTS AND DISCUSSION

Our preliminary results indicated that the content of CYP2E1 protein and transcript in stellate cell preparations was very low compared to that of hepatocytes, and that the apparent CYP2E1 content was dependent upon the degree of hepatocyte contamination in the stellate cell preparations. Consequently, special emphasis was placed on minimizing hepatocyte contamination. In our hands the hepatocyte contamination (hepatocytes per total cell count) varied between 0 and 3%. The influence of hepatocyte contamination on the apparent amount of immunoreactive CYP2E1 protein is depicted in Figs. 1 and 2. We estimate from these data that hepatocytes contain approximately 40 times more immunoreactive CYP2E1 than do stellate cells. Comparison of the amount of CYP2E1 transcripts relative to β-actin mRNA in hepatocyte preparation with that in stellate cell preparations (Figs. 2 and 3) indicated that the difference was at least as large as at the protein level. By plotting the hepatocyte contamination against the relative content of CYP2E1 in stellate cells compared to hepatocytes, significant correlations were, as expected, obtained ($r = 0.74$, $P < 0.05$ for protein and $r = 0.99$, $P < 0.05$ for mRNA) (Fig. 2). Thus, the minor hepatocyte contamination of our stellate cell preparations, typically below 1%, increased considerably the immunoblot signal (Figs. 1, 2) and the RT-PCR signal (Figs. 2, 3) obtained from the stellate cells due to the approximately 10-fold larger cell volume of hepatocyte cells. The levels of CYP2E1 protein in the two purest stellate cell preparations were 2% and 4% of the mean hepatocyte CYP2E1 level, and CYP2E1 mRNA was almost undetectable in a pooled sample (Figs. 2, 3). Extrapolated to 0% hepatocyte contamination with linear regression analysis, the estimated CYP2E1 expression in stellate cells was 4% of that in hepatocytes for protein and 0% for mRNA (Fig. 2).

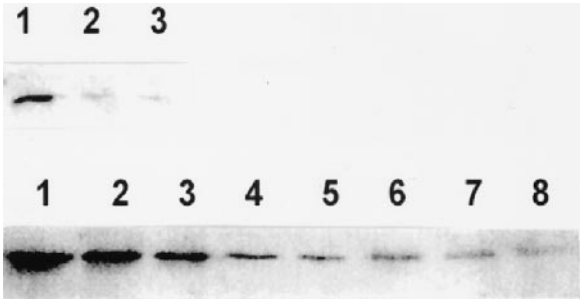

FIG. 1. Immunoreactive CYP2E1 in stellate cell and hepatocyte preparations. Upper panel: 3 μg of stellate cell protein was separated in 9% SDS polyacrylamide gels, electroblotted to nitrocellulose, and probed with rabbit anti-rat CYP2E1 antiserum as described in Materials and Methods. The hepatocyte contamination per total cell count was 3% [1], 1.2% [2], and 0.3% [3]. Lower panel: comparison of CYP2E1 content in hepatocytes vs stellate cells as analyzed by immunoblotting of serial dilution of 0.26, 0.13, 0.06, and 0.03 μg of hepatocyte protein (wells 1–4) and of 2.6, 1.3, 0.65, and 0.33 μg of stellate cell protein (wells 5–8). The hepatocyte sample contained approximately 40 times more CYP2E1 than the stellate cell sample.

During the preparation of this manuscript, the study of Parola *et al.* [17] appeared. These authors investigated human hepatic stellate cells and were unable to detect any immunoreactive CYP2E1 protein. These observations con-

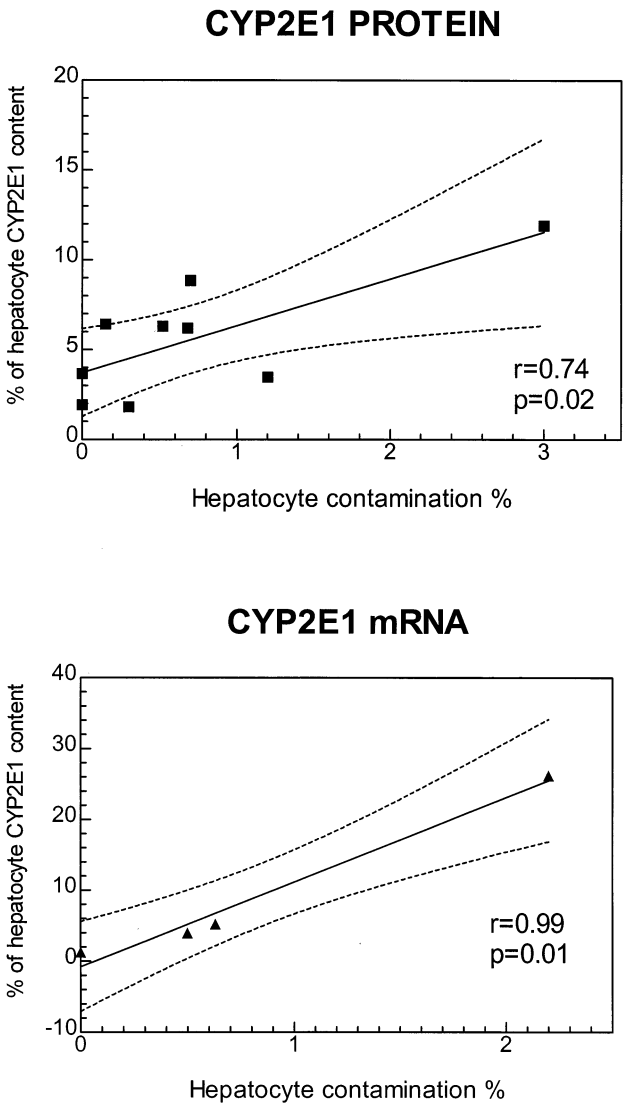

FIG. 2. The effect of hepatocyte contamination on the relative contents of CYP2E1 protein and mRNA in stellate cell preparations. CYP2E1 protein was analyzed by immunoblotting followed by videodensitometry quantitation of the blots, and mRNA by RT-PCR as described in Materials and Methods. The points on the mRNA curve are pools of 1–3 stellate cell preparations (total N = 8), and the mean hepatocyte CYP2E1 expression is calculated from 8 or 9 preparations. Linear regression analysis with the 95% confidence interval and Pearson's correlation coefficient *r* are presented.

trast to those of Yamada *et al.* [10], who reported that the expression of CYP2E1 protein in rat stellate cells was 21% of that in hepatocytes. It is obvious that the purity of the stellate cell preparation is crucial when studying the expression of a typically hepatocyte-expressed gene, and that even a minor hepatocyte contamination will interfere with the analysis of CYP2E1 protein and mRNA. Although Yamada *et al.* reported having used pure stellate cell preparations, even a low hepatocyte contamination in their study may explain the contradicting results in these two studies. It is also possible that there are differences in *CYP2E1* expression at the cellular level between different

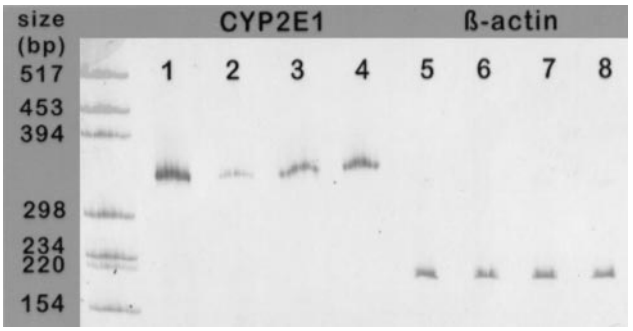

FIG. 3. Comparison of the expression of CYP2E1 and β-actin mRNA in stellate cells and in hepatocytes. CYP2E1 fragments of 311 bp were amplified from 3 μL of cDNA from stellate cell RNA [1, 2] and 0.3 μL of cDNA from hepatocyte RNA [3, 4] of the same animals as described in Materials and Methods. The stellate cell preparations contained 2.2 [1, 5] and 0% [2, 6] hepatocytes of the cell number. One microliter of cDNA was used from both stellate cell [6, 7] and hepatocyte [7, 8] RNA to amplify a 211-bp β-actin fragment. The amplification products were separated on 20% polyacrylamide gel and stained with silver.

animal strains or as a result of different diets. It must also be stressed that analysis and quantitative comparison of signals obtained by immunoblotting are notoriously difficult if the samples contain very different amounts of the antigen. Our finding, that the CYP2E1 transcripts were virtually absent in the purest stellate cell preparations, supports our protein data.

From these data, we conclude that at least in the ethanol-uninduced liver the expression of *CYP2E1* in stellate cells is so low that it is unlikely that CYP2E1-mediated pathways in stellate cells could directly stimulate them to initiate alcohol-induced fibrogenesis.

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