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Detection of Cytochrome P4503A (CYP3A) in Human Hepatic Stellate Cells

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In this study we have investigated the occurrence of cytochrome P450 isoforms and of related cytochrome P450 reductase in human hepatic stellate cells (hHSC), a type of cell having relevant roles in physiopathological conditions of the liver. By performing immunoblotting of hHSC microsomes and immunofluorescence analysis associated to confocal laser microscopy we detected only P450 enzymes belonging to the cytochrome P450 3A subfamily (CYP3A) as well as cytochrome P450 reductase. The presence of CYP3A was further indicated by detection of testosterone 6β-hydroxylase activity in hHSC microsomes. Other important human P450 forms were either undetectable (CYP1A2, CYP2E1, CYP2C8/9/19 and CYP4A) or bearly detectable (CYP1A1) in hHSC. This is the first study showing existence of active cytochrome P450 isoforms in human HSC.

Cytochrome P450 (CYP) is a superfamily of heme proteins carrying out oxidative, peroxidative and reductive metabolism of many structurally different drugs, environmental pollutants and carcinogens (1). Hepatic P450s have also a major role in the metabolism of compounds of physiological relevance, including endogenous steroids, fatty acids, retinoids, leucotrienes, bile acids and biogenic amines (2). The most important human liver CYP isoenzymes involved in xeno- and endobiotics metabolism are those in subfamilies: CYP3A (3A4), CYP2C (2C8, 2C9, 2C19), CYP1A (1A1, 1A2), CYP2E (2E1) and CYP2D (2D6) and are largely localized in the endoplasmic reticulum of many cell type, particularly liver parenchymal cells (3,4). However, several studies have shown that CYPs are present also in Kupffer and sinusoidal endothelial cells, suggesting a possible role of non-parenchymal liver cells in the metabolism of endo- and xenobiotics (5-9).

In recent years much attention has been focused on the physiopathological role of hepatic stellate cells (HSC), also referred to as Ito cells, liver fat-storing cells or lipocytes, which are liver specific pericytes located in the space of Disse in close contact with sinusoidal endothelial cells and hepatocytes (10-14). HSC are known to be the major site of storage and metabolism of vitamin A and retinoids in mammalian liver (10-13) as well as the major source of collagen and extracellular matrix components in the fibrotic liver (14-18). Moreover, these cells represent at the same time a critical target and an effective source for several cytokines, growth factors and other endobiotic substances (14-18).

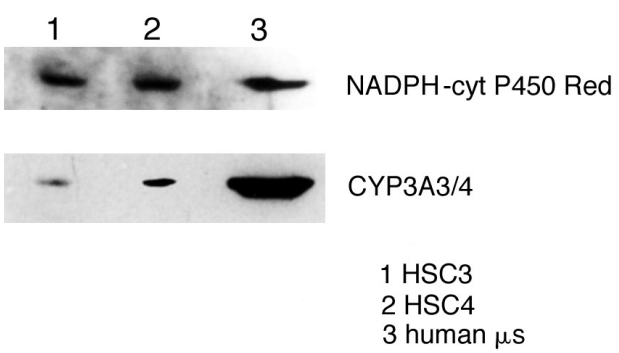

FIG. 1. NADPH-cytochrome P450 Reductase and CYP3A in hHSC. Microsomal proteins (10 μg), prepared from hHSC3 (lane 1), hHSC4 (lane 2) and from human liver samples (1 μg protein, lane 3), were separated by SDS-PAGE and immunoblotted with polyclonal antibodies against CYP3A4 and NADPH-cytochrome P450 reductase. One blot representative of three is shown.

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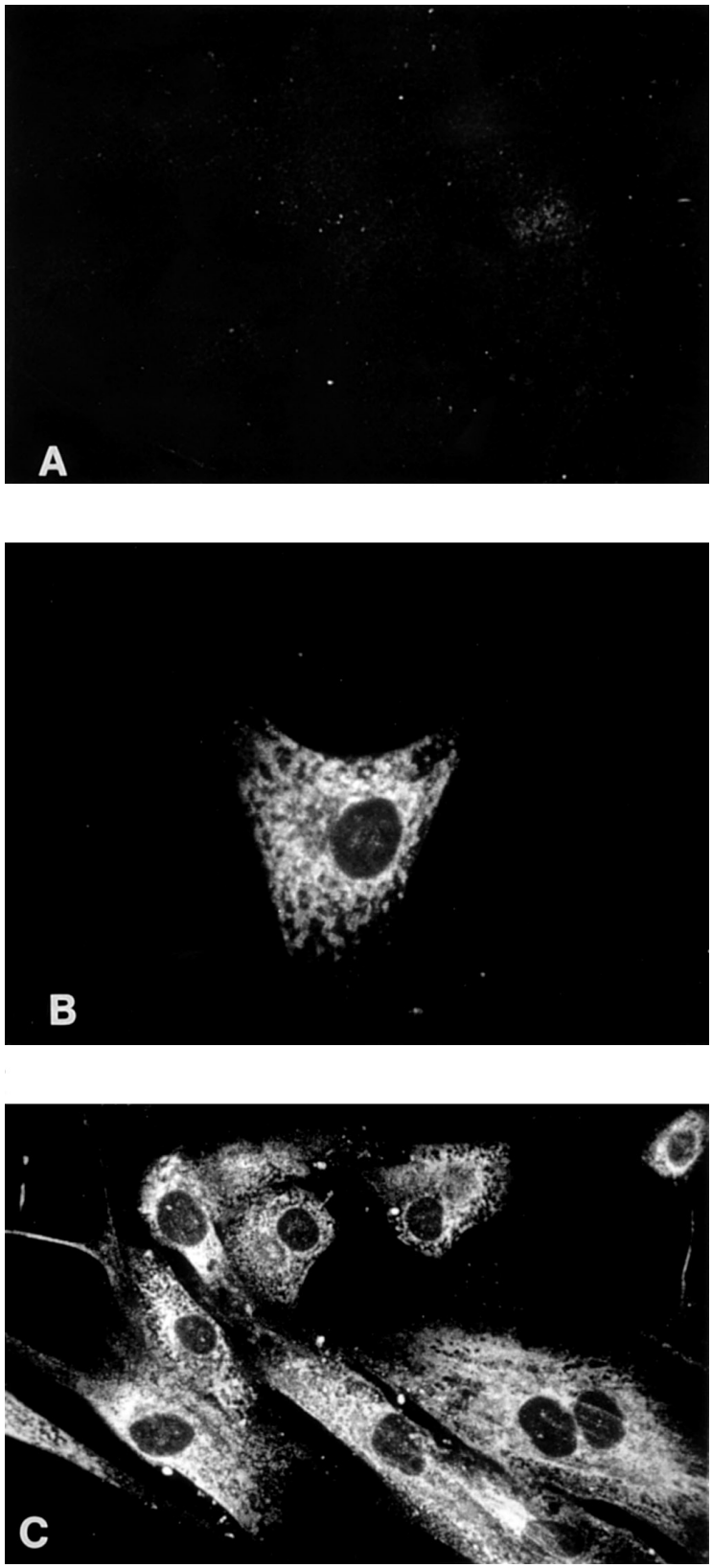

FIG. 2. Intracellular localization of CYP3A4 and cyt-P450 reductase in hHSC, as shown by means of indirect immunofluorescence applied to confocal laser microscopy. hHSC grown on cover slips, fixed and permeabilized were first incubated in the absence of the primary antibody (A) or in the presence of polyclonal antibodies raised against NADPH cyt-P450 reductase (B) or CYP3A (C); cells were then incubated with FITC-conjugated goat anti-rabbit secondary antibodies. Further details are reported in Materials and Methods section.

The important roles of HSC and their localization in the space of Disse have prompted us to investigate the occurrence of major CYP isoforms in HSC isolated from human livers.

MATERIALS AND METHODS

Reagents. Antibodies against the different P450 isoforms were obtained as follows. Polyclonal antibodies against CYP1A1/2 and CYP2C6, cross reacting with CYP2C8, CYP2C9 and CYP2C19 were a kind gift of Prof. Francesco De Matteis (Inst. Pharmacology and Pharmacother., University of Torino, Italy) and dr. Colin Henderson (Biomedical Res. Centre, University of Dundee, U.K.), respectively. Polyclonal antibodies against CYP2E1 and CYP3A4 were raised as previously described (19); antibody against CYP4A (raised in sheep) was kindly provided by Prof. Gordon G. Gibson (University of Surrey, Guildford, U.K.). Appropriated FITC-conjugated antibodies (anti-rabbit and anti-sheep) were obtained from Sigma (Sigma Chem. Co., Milano, Italy) whereas horse-radish peroxidase-conjugated antibodies were purchased from Bio Rad (Milano, Italy).

Cell culture. Human human hepatic stellate cells (hHSC) were isolated from livers unsuitable for transplantation by means of pronase-collagenase digestion and centrifugation on stractan gradients. Isolation procedure and characterization of hHSC were performed as previously described (20,21). The cell were cultured in Iscove's medium supplemented with 20% fetal bovine serum. Microsomal fractions were prepared by ultracentrifugation (22) using hHSC that were maintained in serum free, insulin free (SFIF) Iscove's medium for 24 hrs.

Immunofluorescence analysis. hHSC were cultured on glass cover slips in Iscove's medium until 50-60% confluence and then left in SFIF medium for 24 hrs and processed for indirect immunofluorescence essentially as previously described (23). Briefly, cells were fixed with 4% buffered formalin for 10 min, treated with 0.1% Triton X-100 for 10 min and stained for indirect immunofluorescence using the polyclonal anti CYP isoforms as primary antibody (dilution 1:100 - 1:200 in phosphate buffered saline pH 7.4 containing 0.5% serum bovine albumine) and appropriate FITC-conjugated anti-rabbit or anti sheep antibodies (dilutions 1:250 in phosphate buffered saline pH 7.4 containing 0.5% serum bovine albumin). After repeated washing, the cells were finally mounted in Vectashield (Vector, Milano Italy) and then viewed with a laser scanner confocal microscope Bio Rad MRC 600 equipped with a Nikon diaphot inverted microscope with a 60/1.40 objective. Six focal frames were taken along the z axis at 1 μm intervals and then merged to obtain a reconstructed image.

Western blot. hHSC microsomal proteins were separated by SDS-PAGE and electroblotted on nitrocellulose membranes (Bio Rad Laboratories). Membranes were blocked overnight at 4°C in Tris buffered saline (TBS) containing 5% milk plus 0.05% Tween 20 and then incubated at room temperature with primary and horseradish peroxidase-conjugated secondary antibodies. Detection was carried out by enhanced chemiluminescence (ECL) according to the manufacturer's protocol (Amersham Life Science, Milano, Italy).

Biochemical assay of CYP3A4. Activity of CYP3A4 was evaluated with a radiometric assay monitoring the formation of 6β-hydroxy-testosterone according to a procedure previously described (22). Briefly, the incubation mixture contained 100 μM [4-^{14}C]testosterone (sp. act. 3.75 nCi/nmol), 100 μg protein and 0.5 mM NADPH in 100 mM potassium phosphate buffer, pH 7.4. After a 3 min preincubation at 37°C, the reaction was started by adding NADPH and stopped after 45 min at 37°C with ethyl acetate. Excess substrate was removed and 6-β-hydroxy-testosterone was separated by thin layer chromatography, the band identified using authentic standard, scraped and counted in a liquid scintillation counter.

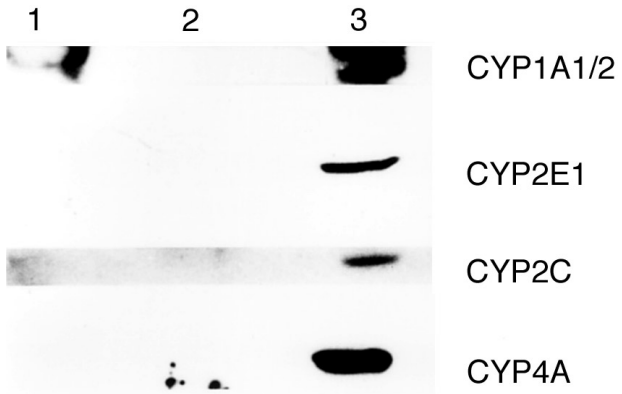

FIG. 3. Western blot analysis of other major P450 isoforms in hHSC. Microsomal proteins (10 μg), prepared from hHSC3 (lane 1), hHSC4 (lane 2) and, for control purpose, from human liver samples (1 μg, lane 3), were separated by SDS-PAGE and immunoblotted with polyclonal antibodies for the different P450 forms.

RESULTS AND DISCUSSION

The presence of CYP isoforms in hHSC was studied in cell cultures obtained from two different human livers designated hHSC3 (from a male donor) and hHSC4 (from a female donor).

Immunoblots of microsomal proteins obtained from both hHSC3 and hHSC4 using a panel of polyclonal antibodies directed against different CYP isoforms demonstrated the presence of significant amounts of CYP3A isoforms (presumably CYP3A4) as well as of NADPH-cytochrome P450 reductase (P450-Red) in the microsomal membranes obtained from both the cell lines (Figure 1). However, microsomes obtained from hHSC3 and hHSC4 did not show any immunodetectable CYP2C, CYP1A2, CYP2E1 or CYP4A (Figure 2). A faint band attributed to CYP1A1 was observed only in one of the cell lines (hHSC3). The presence of CYP3A and P450-Red was confirmed by indirect immunofluorescence analysis using confocal laser microscopy that showed a characteristic spotted intracellular staining typical of a localization in the endoplasmic reticulum. As a matter of fact, the morphological appearance of the staining for CYP3A and P450-Red was clearly overlapping (Figure 3). No detectable immuno-staining of hHSC was obtained using antisera against CYP2C, CYP1A2, CYP1A1, CYP2E1 and CYP4A (data not shown), confirming results obtained by immunoblotting.

The functional activity of CYP3A and P450-red was demonstrated by assay of testosterone hydroxylation in hHSC microsomes. As shown in Table 1, radiometric analysis of 6β-hydroxy-testosterone showed that micro-

TABLE 1

CYP3A4 Catalytic Activity in Human Liver Microsomes and Microsomes from hHSC as Assessed by the Formation of 6β-Hydroxy-testosterone (6β-OH-T)

Sample	Activity (6β-OH-T)	CYP3A4 mean densitometric values per 10 μg protein
hHSC3	5.2 ± 1.5 (3)	2
hHSC4	6.6 ± 1.7 (3)	6
human liver microsomes	608.4 ± 171.9 (5)	509

Note. Results are expressed as pmoles of hydroxylated product / min/mg protein and as means ± SD (n=3-5). The mean densitometric values obtained by evaluating immunodetectable CYP3A4 bands in 3 different SDS-PAGE electrophoresis corrected by taking into account the effective amount of microsomal proteins charged on gels are also shown.

somal fractions prepared from both hHSC lines formed low, but appreciable, amounts of this metabolite when incubated *in vitro* with 0.5 mM NADPH and 100 μM testosterone. CYP3A4 activity in hHSC microsomes was approx. one hundred times lower than the corresponding activity in human liver microsomes. Interestingly, this was consistent with the densitometric analysis of CYP3A4 bands in the Western blots. When using corrections taking into account the respective amount of microsomal proteins used for SDS-PAGE electrophoresis, CYP3A4 appeared to be the only immunodetectable form of CYP in hHSC. Densitometric analysis showed that microsomes obtained from human liver microsomes contained 216 and 84 times, respectively, higher immunodetectable CYP3A4 than hHSC3 and hHSC4 microsomes. This suggests that the related level of CYP3A-dependent testosterone hydroxylase activity and protein detected by Western blotting is the same between microsomes isolated from hHSC and human liver, respectively.

To our knowledge this is the first report concerning expression of specific and active P450 isoforms in hHSC. In humans, the CYP3A subfamily includes the forms CYP3A4, CYP3A5 and CYP3A7, which have more than 84% structural homology (24,25). In human liver, CYP3A4 appears to be expressed to a very high level (26), but up to 30 fold interindividual variations in the hepatic expression of CYP3A4 has been observed (27). CYP3A4 is especially important as it metabolizes a variety of drugs including tricyclic antidepressants, benzodiazepines, dyhydropyridine calcium channel blockers, macrolide antibiotics, cyclosporine and lidocaine. In addition, CYP3A4 mediates the oxidation of several steroids hormones such as dexamethasone, estrogens and testosterone (28,29). In spite of this wide range of activities the function of CYP3A4 in hHSC is at the moment unknown. A role hHSC CYP3A4 in xenobiotic metabolism can not be excluded, but quantitative contribution to the overall hepatic xenobiotic disposure should be not significant. It is much more likely that hHSC CYP3A4 may be important for the metabolism of endogenous compounds, possibly in relation to the relevant role that HSC have in retinoid metabolism (12,30). Further studies are needed to address this point.

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REFERENCES

1. Nelson, D. R., Koymans, L., Kamataki, T., Stegeman, J. J., Feyereisen, R., Waxman, D. J., Waterman, M. R., Gotoh, O., Coon, M. J., Estabrook, R. W., Gunsalus, I. C., and Nebert, D. W. (1996) *Pharmacogenetics* **6,** 1–43.
2. Guengerich, F. P. (1990) *Crit. Rev. Biochem. Mol. Biol.* **25,** 97–153.
3. Gonzales, F. J. (1993) *in* Cytochrome P450 (Schenkman, J. B., and Greim, H., Eds.), pp. 239–257, Springer-Verlag, Berlin, Germany.
4. Shimada, T., Yamazaki, H., Mimura, M., Inui, Y., and Guengerich, F. P. (1994) *J. Pharmacol. Exp. Ther.* **270,** 414–423.
5. Steinberg, P., Lafranconi, W. M., Wolf, C. R., Waxman, D. J., Oesch, F., and Friedberg, T. (1987) *Mol. Pharmacol.* **32,** 463–470.
6. Rich, K., and Lodola, A. (1989) *Cell Biochem. Function* **7,** 275–282.
7. Steinberg, P., Schlemper, B., Molitor, E., Platt, K. L., Seidel, A., and Oesch, F. (1990) *Env. Health Per.* **88,** 71–76.
8. Koop, D. R., Chernosky, A., and Brass, E. (1991) *J. Pharm. Exp. Ther.* **258,** 1072–1076.
9. Koivisto, T., Mishin, V. M., Mak, K. M., Cohen, P. A., and Lieber, C. S. (1996) *Alcohol Clin. Exp. Res.* **20,** 207–212.
10. Wake, K. (1980) *Int. Rev. Cytol.* **66,** 303–353.
11. Blomhoff, R., and Wake, K. (1991) *FASEB J.* **5,** 271–277.
12. Geerts, A., De Bleser, P., Hautekeete, M. L., Niki, T., and Wisse, E. (1994) *in* The Liver: Biology and Pathobiology (Arias, I. M., Boyer, J. L., Fausto, N., Jakoby, W. B., Schachter, D., and Shafritz, D. A., Eds.), pp. 819–838, Raven Press, New York.
13. Pinzani, M., Failli, P., Ruocco, C., Casini, A., Milani, S., Baldi, E., Giotti, A., and Gentilini, P. (1992) *J. Clin. Invest.* **90,** 642–646.
14. Friedman, S. L., Roll, F., Boyles, J., and Bissel, D. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 8681–8685.
15. Bissel, D. M., Friedman, S. L., Maher, J. J., and Roll, F. J. (1990) *Hepatology* **9,** 488–498.
16. Ramadori, G. (1991) *Virchows Archiv B Cell Pathol.* **61,** 147–158.

17. Gressner, A. M. (1991) *Eur. J. Clin. Chem. Clin. Biochem.* **29,** 293–311.
18. Pinzani, M. (1995) *Pharmac. Ther.* **66,** 387–412.
19. Johansson, I., Ekstrom, G., Scholte, B., Puzychi, D., Jornvall, H., Ingelman-Sundberg, M. (1988) *Biochemistry* **28,** 1925–1934.
20. Casini, A., Pinzani, M., Milani, S., Grappone, C., Galli, G., Jezequel, A. M., Schuppan, D., Rotella, C. M., and Surrenti, C. (1993) *Gastroenterology* **10,** 245–253.
21. Marra, F., Valente, A. J., Pinzani, M., Abboud, H. E. (1993) *J. Clin. Invest.* **92,** 1674–1680.
22. Neve, E., Eliasson, E., Pronzato, M. A., Albano, E., Marinari, U., and Ingelman-Sundberg, M. (1996) *Arch. Biochem. Biophys.* **333,** 459–465.
23. Palladini, G., Finardi, G., and Bellomo, G. (1996) *Exp. Cell Res.* **223,** 83–90.
24. Nelson, D. R., Kamataki, T. M., Waxman, D. J., Guengerich, F. P., Estabrook, R. W., Feyereisen, R., Gonzalez, F. J., Coon, M. J., Gunsalus, I. C., Gotoh, O., Okuda, K., and Nebert, D. W. (1993) *DNA Cell Biol.* **12,** 1–51.
25. Gonzalez, F. J., Schmid, B. J., Umeno, M., McBride, O. W., Hardwick, J. P., Meyer, U. A., Gelboin, U. A., and Idle, J. R. (1988) *DNA Cell Biol.* **7,** 79–86.
26. Bork, R. W., Muto, T., Beaune, P. H., Srivastava, P. K., Lloyd, R. S., and Guengerich, F. P. (1989) *J. Biol. Chem.* **264,** 910–919.
27. Wrighton, S. A., Ring, B. J., Watkins, P. B., and Van den Branden, M. (1989) *Mol. Pharmacol.* **36,** 97–105.
28. Brian, W. R., Sari, M. A., Iwasaki, M., Shimada, T., Kaminsky, L. S., and Guengerich, F. P. (1990) *Biochemistry* **29,** 11280–11292.
29. Renaud, J. P., Cullin, C., Pompon, D., Beaune, P., and Mansuy, D. (1990) *Eur. J. Biochem.* **194,** 889–896.
30. Blaner, W. S. (1994) *in* The Liver: Biology and Pathobiology (Arias, I. M., Boyer, J. L., Fausto, N., Jakoby, W. B., Schachter, D., and Shafritz, D. A., Eds.), pp. 529–541, Raven Press, New York.