Differences in adult and foetal human cytochrome *P*-450 forms recognized by monoclonal antibodies with specificity for the P450III family

Tristan S. BARNES,* M. Danny BURKE† and William T. MELVIN*‡

Departments of *Biochemistry and †Pharmacology, University of Aberdeen, Marischal College, Aberdeen AB9 1AS, Scotland, U.K.

Six murine monoclonal antibodies raised against a major human adult liver cytochrome *P*-450 (*P*-450) of the PCN family (P450III) detected a protein in human foetal liver microsomes (microsomal fractions) which had an approx. 1 kDa higher molecular mass on SDS/polyacrylamide-gel electrophoresis than the protein recognized in human adult liver microsomes. Although each of the antibodies recognized both the adult and the foetal forms, antibody HL4 showed higher affinity for the foetal form. Recognition by the monoclonal antibodies of peptides generated by proteolytic cleavage of microsomal proteins showed different patterns for the adult and foetal forms. It is concluded that the foetal *P*-450 form recognized by antibodies to the major human adult liver form P450hA7, although structurally similar, is either a distinct *P*-450 isoenzyme or that the adult and foetal proteins have different covalent modification. Immunoquantification experiments showed comparable levels of the *P*-450 forms in adult and foetal liver, although there appeared to be less inter-individual variation in foetal livers.

INTRODUCTION

The cytochromes *P*-450 (*P*-450), a group of haemo-proteins located predominantly in the endoplasmic reticulum (microsomal fraction) of the liver, are responsible for the oxidation of a wide variety of endogenous substrates and xenobiotics, including drugs, steroids, fatty acids, mutagens and carcinogens (Conney, 1982).

Although the ability of human foetal liver to metabolize drugs and carcinogens has long been known (Pelkonen, 1979), there are few reports of the characterization of individual forms of human foetal *P*-450. Kitada *et al.* (1985*a,b*, 1987*a,b*) have purified P-450HFLa from human foetal liver to a specific content of 12.6 nmol/mg of protein and have shown it to be active in a number of different dealkylation and hydroxylation reactions. P-450HFLa (measured immunochemically) represented 36 % of total human foetal liver *P*-450, but less than 5 % of total human adult liver *P*-450 (the totals being determined spectroscopically). The adult and foetal forms recognized by antiserum against P-450HFLa had an identical M_r on SDS/polyacrylamide-gel electrophoresis (PAGE).

Another human foetal liver *P*-450 has been identified by cross-reactivity with polyclonal antibodies raised against a purified form of human adult liver *P*-450, namely P450_s (Cresteil *et al.*, 1985). The abundance of this form in human foetal livers correlated with aldrin epoxidase, benzphetamine *N*-demethylase and dehydroepiandrosterone 16 α -hydroxylase activities. Immunochemical measurements of P450_s in human foetal liver showed levels 2-fold higher than spectroscopically

determined total *P*-450, but this discrepancy is often found and indicates only that P450_s accounted for a large proportion of the *P*-450 protein in the foetal liver. It would appear that foetal P450_s and P-450HFLa are different, since P450_s is also a major component of total human adult liver *P*-450, whereas P-450HFLa is not. The M_r for P450_s was the same in adult and foetal livers.

We have developed monoclonal antibodies to a major form of human adult liver *P*-450 (Barnes *et al.*, 1987) and have used these to investigate immunorelated *P*-450 forms in human foetal liver microsomes.

MATERIALS AND METHODS

Materials

Post-mortem livers were obtained, with ethical permission, from clinically aborted fetuses within 1 h of the operation or from adult renal-transplant donors within 30 min of circulatory arrest and frozen at -80°C until use. Newborn-calf serum was from Gibco, Paisley, Renfrewshire, Scotland, U.K. Goat anti-mouse Ig conjugated to horseradish peroxidase was from Bio-Rad, and ^{125}I -labelled anti-mouse IgG was from Amersham International. *Staphylococcus aureus* V8 proteinase, papain and 4-chloro-1-naphthol were from Sigma, SDS from Fisons and acrylamide from Serva.

Monoclonal antibodies

The production and characterization of the monoclonal antibodies being used here were described previously (Barnes *et al.*, 1987). Monoclonal antibodies were prepared for use by 45 %-satd. $(\text{NH}_4)_2\text{SO}_4$ pre-

Abbreviations used: PCN, pregnenolone 16 α -carbonitrile; PB, phenobarbitone; PAGE, polyacrylamide-gel electrophoresis; PBS, phosphate-buffered saline (137 mM-NaCl/3 mM-KCl/8 mM- Na_2HPO_4 , pH 7.2); *P*-450, cytochrome *P*-450, TBS, Tris-buffered saline (170 mM-NaCl, 10 mM-Tris/HCl, pH 7.5).

‡ To whom correspondence and reprint requests should be sent.

precipitation of either cell-culture supernatant or ascitic fluid from mice injected with the relevant hybridoma lines.

Preparation of microsomes

Microsomes were prepared as described previously (Burke *et al.*, 1983). Total *P*-450 was measured by CO-difference spectroscopy (Omura & Sato, 1964) and protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Immunoblotting

Human liver microsomal proteins were resolved by discontinuous SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970) using a 7–10% linear-gradient resolving gel. Electroblothing of the resolved proteins on to nitrocellulose paper (Schleicher and Schuell; BA 85; 0.45 μ m pore size) was performed as described by Towbin *et al.* (1979). The nitrocellulose paper was incubated sequentially in: (i) ST buffer (10% newborn-calf serum/0.2% Triton X-100 in PBS) for 30 min; (ii) ST buffer containing monoclonal antibody for 90 min; (iii) ST buffer for 10 min, three times; (iv) ST buffer containing goat anti-mouse IgG conjugated to horseradish peroxidase (1:500); and finally (v) ST buffer for 10 min three times. The nitrocellulose was then rinsed briefly in TBS before staining with freshly prepared 4-chloro-1-naphthol (0.5 mg/ml)/H₂O₂ (0.015%, v/v) in TBS containing 10% (v/v) methanol.

Proteolytic cleavage

Limited proteolysis of human adult and foetal liver microsomes was performed by a modification of the method described by Sesardic *et al.* (1986). A 100 μ g portion of either adult or foetal liver microsomes was diluted to 60 μ l in 250 mM-Tris/HCl, pH 6.8, containing 0.1% SDS, 10% (v/v) glycerol and 0.001% Bromophenol Blue. Either 5 μ g of *S. aureus* V8 proteinase or 0.2 μ g of papain were added and samples were digested at 37 °C for 1, 7 or 20 min before addition of 10 μ l of 50% (v/v) 2-mercaptoethanol containing 10% (w/v) SDS.

Various quantities of digested microsomes were incubated at 100 °C for 3 min and were then subjected to SDS/PAGE using 12.5% (w/v) acrylamide (Laemmli, 1970). This was followed by blotting as described above.

Immunoquantification using monoclonal antibodies

The level of antigen expressed in various samples of liver microsomes was quantified by using an immunoblot procedure modified to accommodate a radioiodinated second antibody. After SDS/PAGE, the resolved protein samples were electroblotted on to nitrocellulose paper which was stained with 0.15% (w/v) Ponceau S in 3% (w/v) TCA for 5 min before rinsing with PBS. The protein bands were clearly visualized and the region known to correspond to *P*-450 was cut out as 8 mm squares. The squares were arranged, in known order, in the wells of 24-well cell-culture plates. After blocking with ST buffer, the nitrocellulose squares were dried at room temperature for 2 h. Instead of the 60 min incubation with the enzyme-linked second antibody, the squares received an overnight incubation in ¹²⁵I-labelled anti-mouse Ig (diluted 1:100 with ST buffer). After the PBS rinse, the radioactivity of the squares was determined

on a Beckman Gamma 5500 counter. A calibration curve of 0–150 ng of the pure P450hA7 was run in every gel. The calibration curve was linear and the value for percentage of microsomal protein was independent of sample size over this range. Backgrounds were 200 c.p.m. from a non-*P*-450 area of the blot and 11750 c.p.m. corresponded to 150 ng of pure P450hA7.

RESULTS

The monoclonal antibodies used in the present study were raised against either semi-purified *P*-450 (antibodies HP3, HP10 and HP16) or crude microsomes (antibodies HL3, HL4 and HL5) from adult human liver and were subsequently found to recognize a highly purified *P*-450 (P450hA7) prepared in our laboratories from adult human liver microsomes (Barnes *et al.*, 1987). P450hA7 was identical in its *N*-terminal sequence over 18 amino acids with human adult *P*-450 HLP (Watkins *et al.*, 1985) and human adult *P*-450_{NF} (Beaune *et al.*, 1986). Each of these antibodies gives a single band of the same *M_r* on immunoblots of liver microsomes from different adult individuals. In contrast, the six antibodies recognize, between them, at least three different adult male rat liver

Fig. 1. Comparison of adult and foetal human liver microsomal proteins

Adult (lane 2; 25 μ g) and foetal (lane 4; 25 μ g) microsomal proteins were resolved by SDS/PAGE and stained with Coomassie Blue. Samples of pure P450hA7 (lane 3; 1 μ g) and standard molecular-mass markers (lane 1; 10 μ g) are also shown.

Fig. 2. Immunoblot of adult and foetal liver microsomal proteins with monoclonal antibodies HP3, HL3 and HP10

Liver microsomal proteins (12.5 μ g) from two adults (lanes 1 and 3) and two foetuses (lanes 2 and 4) were resolved by SDS/PAGE, electroblotted on to nitrocellulose paper and incubated with monoclonal antibodies HL3 (a), HP3 (b) and HP10 (c).

P-450 forms, one of which is constitutive and the others inducible by PCN (Barnes *et al.*, 1987).

A comparison of the Coomassie Blue-stained profiles of adult and foetal human liver microsomal proteins resolved by SDS/PAGE shows the presence of a 53 kDa band in adult microsomes which is absent from foetal microsomes, and the presence of a 54 kDa band in the foetal microsomes which is absent from the adult sample (Fig. 1). On immunoblots each of the monoclonal antibodies recognized a protein of 54 kDa in the foetal liver microsomes and a 53 kDa protein in the adult liver microsomes (Fig. 2). The comparative intensities of staining between the foetal and adult *P*-450 proteins with the monoclonal antibodies fell into three categories; HP3 (and HL4, not shown) stained the foetal form much more strongly than the adult form; HL3 (and HL5, not shown) stained the foetal form a little more strongly than the adult form; and HP10 stained the two forms equally.

Proteolytic cleavage by *S. aureus* V8 proteinase of the adult and foetal human liver microsomal proteins before electrophoresis and immunostaining resulted in partial digestion of the *P*-450 in each case, even though a range of conditions for the proteolysis was employed (Fig. 3).

Fig. 3. Recognition of peptides generated by V8-proteinase cleavage of adult and foetal liver *P*-450 by monoclonal antibodies HL3 and HP3

Adult and foetal liver microsomal proteins were subjected to limited digestion by V8 proteinase as described in the text. The resulting peptides were resolved by SDS/PAGE, electroblotted on to nitrocellulose paper and incubated with monoclonal antibody HL3. 'Adult' peptides were run in lanes 2, 4 and 6; 'foetal' peptides in lanes 1, 3 and 5. Lanes 1 and 2 contained samples digested for 1 min; lanes 3 and 4, samples digested for 7 min; and lanes 5 and 6, samples digested for 25 min. (a) HL3; (b) HP3

The pattern of digestion products was markedly different for the foetal and adult forms when any of the monoclonal antibodies was employed, although the digestion products identified differed for each of the antibodies. Since each of the monoclonal antibodies is known to recognize a different epitope (Barnes *et al.*, 1987), the variation in pattern with antibody used might be expected.

When microsomal proteins were digested with papain, three of the antibodies showed a similar immunoblotting pattern to each other (Fig. 4) in which a single band was identified, but corresponded to a higher molecular mass in foetal than in adult samples. With other antibodies no differences between the adult and foetal samples could be observed.

The amounts of the *P*-450 forms being recognized in adult and foetal human liver microsomes were measured employing 125 I-labelled second antibody to quantify the binding of the monoclonal antibodies HL3 and HL4. Initial experiments (not shown) were performed by dot-blotting, using a calibration curve generated with known quantities of pure adult P450hA7, but it was obvious that the linear range of the calibration curve did not

Fig. 4. Recognition of peptides generated by papain cleavage of adult and foetal liver *P*-450 by monoclonal antibody HL3

Adult and foetal liver microsomal proteins were subjected to limited digestion by papain as described in the text. The resulting peptides were resolved by SDS/PAGE, electroblotted on to nitrocellulose paper and incubated with monoclonal antibody HL3. Lanes 1 and 2 contained undigested samples of adult and foetal liver microsomes respectively. Samples were digested with papain for 1 min (lanes 3 and 4); for 7 min (lanes 5 and 6) and for 25 min (lanes 7 and 8). 'Adult' peptides were run in lanes 3, 5 and 7; 'foetal' peptides were run in lanes 4, 6 and 8.

correspond to the linear range for the microsomal samples, probably because of the presence of the many extra proteins in microsomes. Consequently, the method was modified by first separating the *P*-450 proteins from other proteins by SDS/PAGE and then electroblotting to nitrocellulose. The region of nitrocellulose corresponding to *P*-450 was then cut out and incubated with monoclonal antibody followed by ^{125}I -labelled second antibody and the radioactivity measured. In this way linearity of radioactivity against protein level was obtained, not just for the pure P450hA7, but also for the microsomal samples. By using antibody HL4, apparently higher levels of immunoreactive *P*-450 were found in foetal livers than in adult livers (Fig. 5). However, when antibody HL3 was used, the levels of immunoreactive *P*-450 measured in foetal liver were significantly decreased and were comparable with those in adult livers. This difference in behaviour is attributable to different

affinities of HL4 for the adult and foetal forms (Fig. 6), but similar affinities of HL3 for the two forms. The affinity of HL4 for the foetal form was higher, even though HL4 was raised against adult liver microsomes.

DISCUSSION

The small, but reproducible, difference in M_r on SDS/PAGE between adult and foetal human liver *P*-450 forms recognized by the same monoclonal antibodies has not previously been reported. Furthermore, the M_r value is not the only difference found, since antibodies HL3 and HL4 differed in the affinity of their reactions with the adult and foetal *P*-450 forms respectively (Figs. 5 and 6), and the products of proteolytic digestion were different (Figs. 3 and 4). A further difference between adult and foetus is that the pattern of immunohistochemical staining is centrilobular with adult human liver but pancreatic with human foetal liver, using any of the six antibodies (Murray *et al.*, 1987).

Since these findings show the presence of different, major, immunorelated forms of *P*-450 in foetal and adult human liver respectively, it will be important to identify where the differences reside. It is possible that the foetal and adult *P*-450 variants will be found to differ in activity as well as structure: this has implications for foetal drug metabolism and consequently for predicting the foetal effects of maternal exposure to drugs and environmental and dietary chemicals during pregnancy. It may also be relevant that the hydroxylation of dehydroepiandrosterone correlated with P450₅ concentration and was more active in foetal than adult microsomes, whereas the hydroxylation of acetanilide showed a correlation with P450₅ in adult microsomes, but not in foetal microsomes (Cresteil *et al.*, 1985).

Most *P*-450 forms have very similar subunit molecular masses on SDS/PAGE (48–57 kDa) and therefore the fact that the difference in M_r between the immunodetected foetal and adult forms of *P*-450 was small is no indication of the extent of structural similarity between them. However, the recognition of both adult and foetal forms by all the monoclonal antibodies tested suggests a high degree of sequence similarity. Expression of different *P*-450 forms may result either from altered processing of precursor RNA or by expression of a different gene. *N*-Terminal sequencing suggests that P450hA7 is highly related to the human P-450HLp and P-450_{NF} forms, and the use of probes for these forms in Southern hybridization analysis indicates that there may be three to five related genes in this *P*-450 family in humans (Molowa *et al.*, 1986; Beaune *et al.*, 1986). In the rat, two *P*-450 forms belonging to the same P450III gene family (Nebert *et al.*, 1987) have been clearly identified by molecular cloning; P450PCN1 is induced by PCN, dexamethasone and phenobarbitone and is not found in control rats at any age; the level of the structurally similar, phenobarbitone-inducible (but not PCN-inducible) P450PCN2 reaches a peak in females at 2 weeks of age, but continues to increase in males until 12 weeks, greatly surpassing the levels in females (Gonzalez *et al.*, 1986). In contrast, despite our antibodies recognizing highly purified rat *P*-450 forms showing *N*-terminal sequence similarity with P450PCN1 and P450PCN2 (results not shown), we saw no consistent sex difference in the level of the 53 kDa protein in adult human liver identified with our antibodies to P450hA7,

Fig. 5. Immunoquantification of immunoreactive human *P*-450 in liver microsomes from 12 adult individuals and three fetuses

Human liver microsomes were subjected to SDS/PAGE and electroblotted on to nitrocellulose paper. Nitrocellulose squares (8 mm × 8 mm), each containing one immunoreactive P450 band, were cut and incubated with monoclonal antibodies HL3 (■), HL4 (■) and HL5 (□). Bound antibody was measured using ¹²⁵I-labelled second antibody. Calculation of the immunoreactive *P*-450 levels in adult (A1-12) and foetal (F1-3) human liver microsomes was performed using a calibration curve of 0–150 ng of the pure P450hA7 in every gel. The calibration curve was linear, and the value for percentage of microsomal protein was independent of sample size over the range of the calibration curve.

Fig. 6. Comparison of the affinity of monoclonal antibody HL4 for adult and foetal immunoreactive *P*-450

Adult and foetal human liver microsomes (12.5 μg) were subjected to SDS/PAGE and electroblotted on to nitrocellulose paper. Nitrocellulose squares (8 mm × 8 mm), each containing one immunoreactive *P*-450 band, were cut out and incubated with serial dilutions of monoclonal antibody HL4 using ¹²⁵I-labelled second antibody to measure binding. Since the total bound radioactivity was different for each sample, results are expressed as a percentage of maximum binding for microsomes from two adult individuals (●, ○) and two fetuses (■, □).

nor any firm evidence of the presence of another immunochemically related *P*-450. However, it is possible that our antibodies are capable of recognizing different *P*-450 proteins which are highly related in sequence and have the same M_r . For example, protein sequences predicted from the *P*-450HLP and *P*-450_{NF} sequences only differ in eight positions.

The possible relationship between the foetal *P*-450 form identified here and human foetal liver *P*-450HFLa purified by Kitada *et al.* (1985a) is confused. *P*-450HFLa accounted for 36% of foetal liver *P*-450 and less than 5% of adult liver *P*-450, but in our experiments similar levels of the immunoquantified *P*-450 were seen in both adult and foetal livers. Moreover, *P*-450HFLa had an identical M_r in adult and foetus. On the other hand, *P*-450HFLa has recently been shown to have some *N*-terminal sequence similarity with *P*-450_{NF} in positions 3–10 (Kitada *et al.*, 1987b).

Our results (Fig. 5) also highlight a problem in the interpretation of data obtained by immunoquantification of proteins in a system as complex as *P*-450. Given the number of structurally highly similar, yet different, *P*-450 proteins, it is possible that an antibody will recognize several *P*-450 forms, but with different affinities. This appears to be the reason why our two antibodies, HL3 and HL4, gave different values for the quantification of foetal *P*-450 (obtained using a calibration curve for adult *P*450hA7). It will probably be important in future immunoquantification experiments to ensure that the antibody recognizes only one *P*-450 form in the tissue under study and that the calibration is performed with the identical form.

This work was supported by the Science and Engineering Research Council, the Scottish Home and Health Department and the Aberdeen University Advisory Committee for Research. We are grateful to Mr. J. Engeset (Department of Surgery), Professor J. C. Petrie (Department of Medicine and Therapeutics) and Dr D. Abramovich (Department of Obstetrics and Gynaecology) for providing human livers.

REFERENCES

- Barnes, T. S., Shaw, P. M., Burke, M. D. & Melvin, W. T. (1987) *Biochem. J.* **248**, 301–304
- Beaune, P. H., Umbenhauer, D. R., Bork, R. W., Lloyd, R. S. & Guengerich, F. P. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 8064–8068
- Burke, M. D., Falzon, M. & Milton, A. S. (1983) *Biochem. Pharmacol.* **32**, 389–397
- Conney, A. H. (1982) *Cancer Res.* **42**, 4875–4917
- Cresteil, T., Beaune, P., Kremers, P., Celier, C., Guengerich, F. P. & Leroux, J. P. (1985) *Eur. J. Biochem.* **151**, 345–350
- Gonzalez, F. J., Song, B.-J. & Hardwick, J. P. (1986) *Mol. Cell Biol.* **6**, 2969–2976
- Kitada, M., Kamataki, T., Itahashi, K., Rikihisa, T., Kato, R. & Kanakubo, Y. (1985a) *Arch. Biochem. Biophys.* **241**, 275–280
- Kitada, M., Kamataki, T., Itahashi, K., Rikihisa, T., Kato, R. & Kanakubo, Y. (1985b) *Biochem. Biophys. Res. Commun.* **131**, 1154–1159
- Kitada, M., Kamataki, T., Itahashi, K., Rikihisa, T. & Kanakubo, Y. (1987a) *Biochem. Pharmacol.* **36**, 453–456
- Kitada, M., Kamataki, T., Itahashi, K., Rikihisa, T. & Kanakubo, Y. (1987b) *J. Biol. Chem.* **262**, 13534–13537
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Molowa, D. T., Schuetz, E. G., Wrighton, S. A., Watkins, P. B., Kremers, P., Mendez-Picon, G., Parker, G. A. & Guzelian, P. S. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5311–5315
- Murray, G. I., Barnes, T. S., Sewell, H. F., Ewen, S. W. B., Melvin, W. T., Shaw, P. M., Fowler, J. & Burke, M. D. (1987) *Histochem. J.* **19**, 537–545
- Nebert, D. W., Adesnik, M., Coon, M. J., Estabrook, R. W., Gonzalez, F. J., Guengerich, F. P., Gunsalus, I. C., Johnson, E. F., Kemper, B., Levin, W., Phillips, I. R., Sato, R. & Waterman, M. R. (1987) *DNA* **6**, 1–11
- Omura, T. & Sato, R. (1964) *J. Biol. Chem.* **239**, 2370–2378
- Pelkonen, O. (1979) in *The Induction of Drug Metabolism* (Estabrook, R. W. & Lindenlaub, E., eds.), pp. 507–516, F. K. Schattauer Verlag, Stuttgart
- Sesardic, D., Boobis, A. R., McQuade, J., Baker, S., Lock, E. A., Elcombe, C. R., Robson, R. T., Hayward, C. & Davies, D. S. (1986) *Biochem. J.* **236**, 569–577
- Towbin, H., Staehelin, T. & Gordon, J. O. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- Watkins, P. B., Wrighton, S. A., Maurel, P., Schuetz, E. G., Mendez-Picon, G., Parker, G. A. & Guzelian, P. S. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6310–6314