J. Steroid Biochem. Molec. Biol. Vol. 43, No. 6, pp. 515–522, 1992
Printed in Great Britain.

0960-0760/92 $5.00 + 0.00

IMMUNOCHEMICAL SPECIFICITY OF PLACENTAL NADPH CYTOCHROME *c* (*P*-450) REDUCTASE IN NEOPLASTIC AND NON-NEOPLASTIC HUMAN TISSUE

D. A. WILLIAMS, A. PUROHIT and M. J. REED*
Unit of Metabolic Medicine, St Mary's Hospital Medical School, Imperial College of Science, Technology and Medicine, University of London, London W2 1PG, England

(Received 13 January 1992)

Summary—NADPH cytochrome *c* (*P*-450) reductase was purified from human placental microsomes using a combination of affinity and gel filtration chromatography. Affinity chromatography using agarose-hexane-adenosine 2′5 diphosphate resulted in two protein bands being detected by SDS–PAGE of approximate M_ws 68 and 75 kDa. Fractions containing the two proteins were pooled, and then resolved using Sephacryl S-200. Both of the purified proteins displayed enzyme activity, measured by their ability to reduce cytochrome *c*. The 75 kDa protein obtained was used to immunize three female New Zealand white rabbits. The IgG fraction was partly purified from rabbit sera which suppressed placental microsomal NADPH cytochrome *c* reductase activity by > 80% using 33% ammonium sulphate. The procured antibody suppressed androstenedione aromatase activity in microsomal preparations of human placental and breast adipose tissue, and NADPH cytochrome *c* reductase activity in prostate (benign and malignant), MDA-MB-231 breast cancer cells, breast adipose, Hep G2 hepatoma cells and placental microsomal preparations. The extent of NADPH cytochrome *c* reductase inhibition varied in the order of malignant prostate < benign prostate < MDA < breast adipose < Hep G2 < placenta. The results suggest that human placental NADPH cytochrome *c* (*P*-450) reductase shares common antigenic epitopes pertinent to its capability of reducing cytochrome *c* in all of the above-mentioned tissues. In attempting to associate possible changes in NADPH cytochrome *c* reductase activity imposed by neoplasia to the obtained immunochemical cross reactivity and enzyme activity results, it was noted that microsomes obtained from MDA cells exhibited enzyme activity significantly less than that of breast adipose microsomes (1.6 and 8.1 nmol/min/mg protein, respectively) and by comparison showed 6% less homology towards the placental antibody. The results obtained for benign and malignant prostate showed no significant difference between the neoplastic states as adjudged by enzyme activity and immunochemical assays.

INTRODUCTION

NADPH cytochrome *c* (*P*-450) reductase is a membrane bound flavoprotein responsible for the catalysis of electron transfer from NADPH to cytochrome *P*-450 via FAD and FMN [1]. The enzyme plays an important role in the detoxification of xenobiotics [2], the activation of procarcinogens [3], as well as in the oxidative metabolism of numerous endogenous compounds such as eicosanoids and steroids [4]. Although several forms of cytochrome *P*-450's, differing in primary structure, substrate and immunochemical specificity have been found in human tissues [5], there is little evidence to suggest the existence of more than one form of NADPH cytochrome *c* (*P*-450) reductase. Immunological studies by Hiwatashi and Ichikawa [6] have shown that NADPH cytochrome *c* (*P*-450) reductase in beef adrenocortical microsomes differs from that of the liver enzyme, while Osawa *et al.* [7] and Guengerich *et al.* [8] have shown that structural differences exist between human NADPH cytochrome *c* (*P*-450) reductase and NADPH cytochrome *c* (*P*-450) reductase found in other species.

The purpose of our study was to purify NADPH cytochrome *c* (*P*-450) reductase from human placental microsomes, obtain polyclonal antibodies to the purified enzyme and to use them to assess whether the enzyme shared common antigenic structures, pertinent to its activity, with NADPH cytochrome *c* (*P*-450) reductase in other human tissues. The procured antibodies were also used in an attempt to detect any structural or/and conformational changes

*To whom correspondence should be addressed.

that might have occurred during the progression of the neoplastic process in some of the tissues studied.

EXPERIMENTAL

Materials

[1β-^{3}H]androstenedione (27.4 Ci/mmol) was supplied from New England Nuclear Corporation (Boston, MA). Adenosine 2′ monophosphoric acid (2 AMP), phenylmethanesulphonyl fluoride (PSMF), FMN, dithiothreitol, sodium cholate, sodium deoxycholate, lubrol PX, cytochrome *c* (horse heart) and nicotinamide adenine dinucleotide phosphate reduced form (tetrasodium salt) came from Sigma Chemical Company (Poole, Dorset, England). Agarose-hexane-adenosine 2′5 diphosphate and sephacryl S-200 were purchased from Pharmacia Ltd. (Milton Keynes, Bucks., England). Glycerol, EDTA (disodium salt) were obtained from Fisons (Loughborough, Leics., England). Minimum essential medium, Eagle (modified) was obtained from Flow Labs (Irvine, Scotland). Fetal calf serum was supplied by Gibco (Paisley, Scotland).

Preparation of placental microsomes

Placentae were normally obtained within 1 h of delivery and placed directly on ice. All procedures were carried out at 4°C. Placentae were initially separated from umbilical cord, connective tissue and large vessels. Excess and coagulated blood were rinsed from the tissue using 3 × 150 ml vol of 0.9% NaCl. The tissue was blotted dry and weighed. The tissue was minced in 50 mM phosphate buffer (pH 7.7) containing 0.25 M sucrose and 0.1 mM EDTA (1 ml for every 2 g tissue) with scissors and then placed in a Waring blender and homogenized for 2 × 20 s interspersed with 1 min cooling. The tissue was further homogenized using an ultra turrax for a further 2 min. The resulting homogenate was then centrifuged at 800 *g* for 20 min. The supernatant was decanted and centrifuged at 15,000 *g* for 30 min. The supernatant was carefully removed from the mitochondrial pellet and further centrifuged at 100,000 *g*. The supernatant from this centrifugation was discarded and the microsomal pellet re-suspended in 100 mM phosphate buffer (pH 7.7) containing 0.1 mM EDTA and re-centrifuged for a further hour at 100,000 *g*. The supernatant was again discarded and each pellet re-suspended in approx. 2–3 ml of 100 mM phosphate buffer (pH 7.7) containing 0.1 mM EDTA, 1 mM dithiothreitol, 5 mg/l butylated hydroxytoluene, 20% (w/v) glycerol and stored at −70°C.

Purification of NADPH cytochrome c (P-450) reductase

The procedures were carried out at 4°C. Microsomal membrane vesicles were thawed and diluted to a protein concentration of 2 mg/ml using 10 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA, 1 mM dithiothreitol 2 μM FMN, 0.4 mM PMSF and 20% glycerol. A 20% (w/v) solution of sodium cholate was added dropwise with gentle stirring to a final concentration of 0.6% (w/v). After stirring for a further 25 min, the sample was centrifuged for 1 h at 100,000 *g*. The supernatant from this centrifugation was then loaded on to a 1 × 6 cm column of agarose-hexane-adenosine 2′5 diphosphate (flow rate 24 ml/h) which had been pre-equilibrated with 10 mM Sorensens phosphate pH 7.0 containing 0.1 mM EDTA and 20% glycerol. The column was then washed and eluted according to a modified procedure adopted by Guengerich and Martin [9]. The column was initially washed with 375 ml 0.1 M phosphate buffer (pH 7.7) containing 0.1 mM EDTA, 0.1% lubrol PX and 20% glycerol, then with 620 ml 30 mM phosphate buffer (pH 7.7) containing 0.1 mM EDTA, 0.15% (w/v) sodium deoxycholate and 20% glycerol (flow rate 36 ml/h). The reductase was eluted with 30 ml of 30 mM phosphate buffer (pH 7.7) containing 0.1 mM EDTA, 0.15% (w/v) sodium deoxycholate, 5 mM 2′AMP, 0.4 mM PMSF and 10% (w/v) glycerol (flow rate 54 ml/h). This solution was dialysed against two changes (2 × 500 ml) of 30 mM phosphate buffer (pH 7.7) containing 0.1 mM EDTA and 10% (w/v) glycerol over a period of 6 h. The dialysed solution was concentrated using an Edwards Modulyo EF4 freeze drier for a period of 8 h and applied in 2 ml portions to a 1.6 × 45 cm column of sephacryl S-200 pre-equilibrated and eluted with 100 mM phosphate buffer (pH 7.7) containing 0.1 mM EDTA and 20% glycerol (flow rate 3.6 ml/h, fraction size 0.45 ml). Absorbances of each fraction were measured at 280 nm using a Pye Unicam SP8-400 uv/vis spectrophotometer.

SDS–PAGE

SDS–PAGE was performed according to the method of Laemmli [10] using 8% gels.

Molecular weight markers were obtained from Sigma and included ovalbumin (43 kDa), phosphorylase *b* (97 kDa) and *β* galactosidase (116 kDa).

Antibody procurement

Antibodies to the purified 75 kDa form of NADPH cytochrome *c* (*P*-450) reductase, were raised in female New Zealand white rabbits. Rabbits were injected with 100 μg of purified protein in Freund's complete adjuvant and boosted with 100 μg protein in Freund's incomplete adjuvant in weeks 2 and 5 and approx. 25 μg in weeks 11, 13 and 15. The IgG fraction was partly purified from the final rabbit bleed (week 16) sera exhibiting > 80% reductase inhibition with 10 μl of sera by selective precipitation using 33% ammonium sulphate [11]. The precipitated protein was dissolved in 0.1 M phosphate buffer (pH 7.7) containing 0.1 mM EDTA.

Tissue and cell preparations

Breast adipose tissue was obtained from 1 patient (aged 17 years) undergoing reduction mammoplasty, microsomes from this tissue being prepared as stated for placental tissue. Prostate tissue was obtained by trans-urethral resection (the status of the tissue being verified by histological methods). Benign prostate tissue from three patients (aged 63 to 76 years) were combined and homogenized together. Malignant prostate obtained from 1 patient (aged 78 years) was separately prepared. Prostate tissue was homogenized using a polytron in ice-cold 50 mM phosphate buffer (pH 7.7) containing 0.25 M sucrose and 0.1 mM EDTA (1 g tissue/4 ml buffer). The homogenate was centrifuged at 15,000 ***g*** for 30 m. The supernatant from this centrifugation was carefully removed from the resulting pellet using a glass pipette and re-centrifuged for 1 h at 100,000 ***g***. The supernatant was discarded and the microsomal pellet so formed re-suspended in a minimum volume of 100 mM phosphate buffer (pH 7.7) containing 0.1 mM EDTA, 1 mM dithiothreitol, 5 mg/l butylated hydroxytoluene, 20% (w/v) glycerol and stored at −70°C until required.

Hep G2 hepatoma and MDA-MB-231 breast cancer cells were cultured in Minimum essential medium, Eagle (modified) with Earl's Salts and 20 mM Hepes buffer and supplemented with 2 mM L-glutamine, 10 mM sodium hydrogen carbonate 1%, non-essential amino acids, 5% fetal calf serum and grown until fully confluent. Cells were removed from the surface and culture flasks using a rubber policeman. Microsomes for both Hep G2 and MDA cells were prepared as stated for prostate tissue.

Enzyme assays and antisera incubations

NADPH cytochrome *c* (*P*-450) reductase activity was assayed by determining the rates of cytochrome *c* reduction at 25°C by addition of the enzyme to a mixture of 0.3 M phosphate buffer (pH 7.7) containing 40 nmol cytochrome *c* (horse heart), 0.1 μmol EDTA and 0.1 μmol NADPH in a total volume of 1 ml [12]. The rate of cytochrome *c* reduction was determined spectrophotometrically at 550 nm using an extinction coefficient of 21 $mM^{-1}\,cm^{-1}$. Aromatase activity was assessed by quantifying the ^3H_2O released from [1β-^{3}H]androstenedione. An aliquot of microsomal protein was incubated with [1β-^{3}H]androstenedione (150 pmol, 10^6 cpm/assay), 1 mM NADPH, 0.1 M phosphate buffer (pH 7.7) at 37°C in a total volume of 0.5 ml for between 0.4 to 3 h with shaking in air. Reactions were terminated and specific activities calculated as described elsewhere [13].

The effects of the raised antibody on NADPH cytochrome *c* reductase and aromatase activity was assessed by pre-incubating microsomal protein and varying concentrations of IgG for 1 h at 25°C. The incubation mixtures were adjusted with pre-immune IgG so that all contained equivalent concentrations of IgG. All assays were performed in triplicate. Inhibition of both NADPH cytochrome *c* reductase and aromatase activities were used as a positive identification of immunological similarity between the tissues investigated.

Protein assay

Microsomal protein concentrations were measured by the methods of Lowry *et al.* [14].

RESULTS

Purification of human placental NADPH cytochrome c (P-450) reductase

Table 1 shows the yield and purification factor obtained by the purification of NADPH cytochrome *c* (*P*-450) reductase from human placental microsomes. All purification steps were performed within 4 days. The purity of the 2′5 ADP agarose fractions were assessed by SDS–PAGE (Fig. 1). Fractions containing the two protein bands detected were further

Table 1. Purification of NADPH cytochrome *c* (*P*-450) reductase from human placental microsomes

Stage of purification	Total protein (mg)	Specific activity (μmol/min/mg)	Yield %	Purification factor
1. Washed microsomes	1505.0	0.018	100.0	1.0
2. Cholate extract	1023.0	0.02	113.0	1.7
3. 2′5 ADP agarose	63.0	3.4	79.1	189.0
4. Sephacryl S-200				
75 kDa form	0.38	3.9	5.6	216.7
68 kDa form	0.47	5.7	9.9	316.7

resolved using a calibrated Sephacryl S-200 column to give two proteins with approximate M_ws of 68 and 75 kDa. Both proteins possessed NADPH cytochrome *c* reductase activity. A typical elution profile of the Sephacryl S-200 column is shown in Fig. 2.

Cross-reactivity of human placental NADPH cytochrome c (P-450) reductase antibodies with non-placental NADPH cytochrome c (P-450) reductase and aromatase.

Rabbit antibodies against the 75 kDa human placental NADPH cytochrome *c* (*P*-450) reductase were successfully raised in 2 out of the 3 rabbits used for immunization. The effects of this placental NADPH cytochrome *c* (*P*-450) reductase antibody on reductase and aromatase activities is shown graphically in Figs 3 and 4. The results from Table 2 show that the antibody procured strongly inhibited, in a dose-dependent manner, NADPH cytochrome *c* reductase activity and to a greater extent aromatase activity in placental microsomes. Due to lack of tissue, aromatase activity inhibition assays using the antibody were not carried out on all the tissue types utilized in this study.

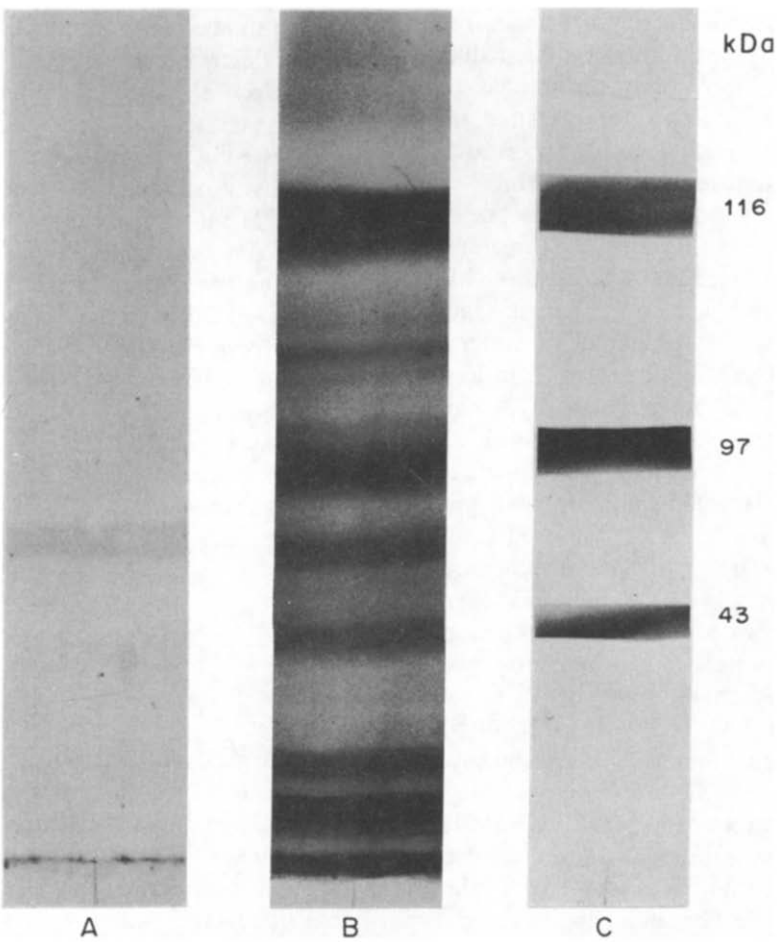

Fig. 1. SDS-PAGE of NADPH cytochrome *c* (*P*-450) reductase. Lane A: reductase purified by affinity gel 2′5 ADP agarose (2 μg). Lane B: cholate soluble fraction of placental microsomes (60 μg). Lane C: molecular weight markers.

Table 2. Cross-reactivity of human placental NADPH cytochrome *c* (*P*-450) reductase IgG with placental and non-placental tissues

Enzyme preparation	Initial NADPH cytochrome *c* reductase activity (nmol/min/mg)	Maximum inhibition by IgG (%)
1. Placental microsomes	19.2	88
2. Breast adipose microsomes	8.1	75
3. (a) Prostate benign microsomes	5.0	65
(b) Prostate malignant microsomes	6.1	64
4. MDA microsomes	1.6	69
5. Hep 62 microsomes	5.6	80
	Initial aromatase activity	
1. Placental microsomes	12 pmol/min/mg	91
2. Breast adipose microsomes	1.1 fmol/min/mg	83

The extent of NADPH cytochrome *c* reductase activity suppression by the antibody varied depending on the type of tissue.

DISCUSSION

Proteolytic cleavage of rabbit liver NADPH cytochrome *c* (*P*-450) reductase has revealed that the enzyme is composed of a large hydrophilic catalytic domain and a smaller hydrophobic amino terminal domain [15]. The proteolytically cleaved lower molecular weight protein is unable to transfer electrons from NADPH to cytochrome *P*-450 [16] but can catalyse the reduction of cytochrome *c* [17]. The proportion of the 68 kDa peptide to that of the 75 kDa in the placental microsomal samples used showed a significantly higher concentration in favour of the 68 kDa protein. This suggests that either the placentae contained a relatively high proportion of trypsin-like proteases and/or that the purification procedures used increased the susceptibility of proteolytic attack, possibly due to the intermittent storage of fractions during the purification stages.

The rabbit IgG fractions obtained were used to determine whether the NADPH cytochrome *c* (*P*-450) reductase antibody from human placenta cross-reacted with the enzyme in microsomal preparations of breast adipose tissue, prostate tissue (benign and malignant), MDA and Hep G2 cells. The IgG isolated inhibited NADPH cytochrome *c* reductase activity in all the tissues investigated, but the degrees of potency varied. Although the assays used are useful in determining whether the enzyme in the various tissues considered share common antigenic structures, as indeed the results suggest, the specificity of antibodies dictated by unknown antigenic determinants makes an attempt at correlating the extent of enzyme inhibition with possible structural or conformational differences difficult. The significance of differences in potency can only properly be explained by the amino acid sequencing and determination of the three dimensional structure of the enzyme in each individual tissue. The recent complete sequencing of NADPH cytochrome *c* (*P*-450) reductase from human liver by Haniu *et al.* [18] has

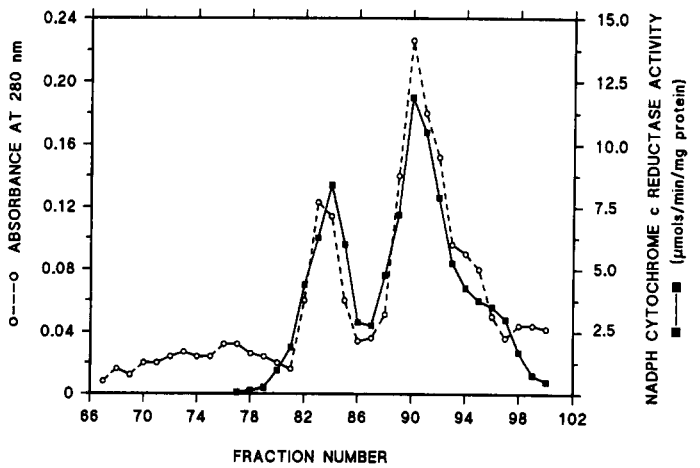

Fig. 2. Sephacryl S-200 column elution profile of the affinity column purified NADPH cytochrome *c* reductase active fractions.

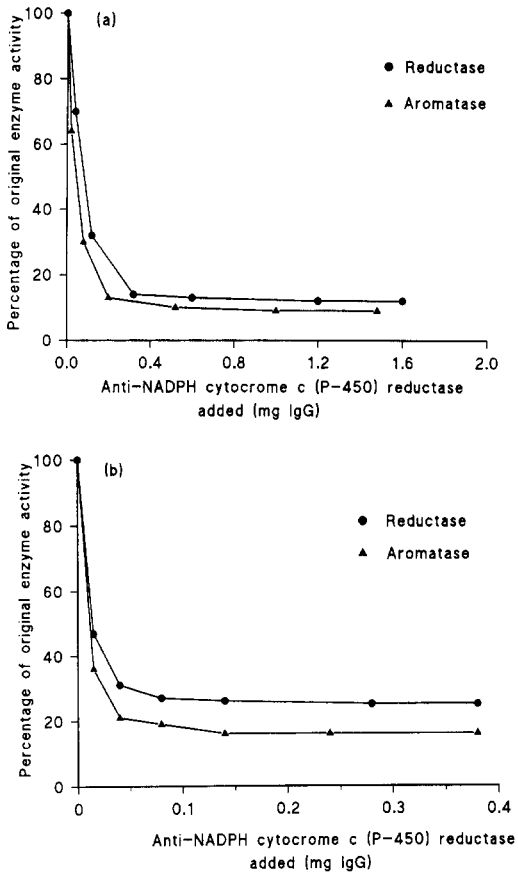

Fig. 3. Inhibition of NADPH cytochrome *c* reductase and aromatase activities in (a) human placental microsomes and (b) breast adipose microsomes, by placental NADPH cytochrome *c* (*P*-450) reductase IgG.

complemented previous immunological studies regarding the enzyme's differentiation between species. However, if the lower immunochemical cross-reactivity of benign prostate, malignant prostate and MDA microsomes is considered in comparison to placental microsomes with the placental NADPH cytochrome *c* (*P*-450) reductase antibody found in this study (Table 2), there may be some justification in assuming that the amino acid composition and/or conformation of this enzyme in placenta is not identical with the neoplastic tissues considered in this study.

NADPH cytochrome *c*(*P*-450) reductase is a required component of human microsomal aromatase [19], catalysing the transfer of electrons from NADPH to cytochrome *P*-450 aromatase. The role which aromatase plays in the conversion of androgens to oestrogens is thought to be of particular importance in the control of oestrogen dependent cancers. *In vivo* experiments have shown that the administration of phenobarbitol, dexamethasone, pregnenolone, 16 carbonitrile and dehydroepiandrosterone to rats increases liver NADPH cytochrome *c* (*P*-450) reductase activity by increasing flavoprotein content [20 and references therein]. However, whether the conformation or indeed the composition of the protein remains unaltered as a result of this stimulation is unclear. A number of reports have stated that aromatase activity in breast

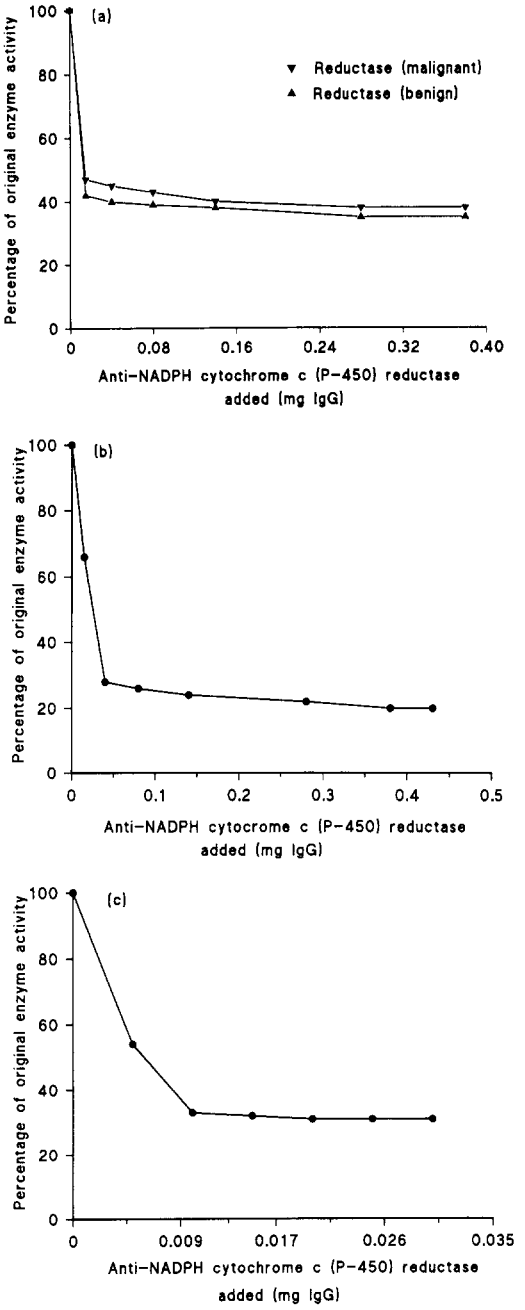

Fig. 4. Inhibition of NADPH cytochrome *c* reductase activity in (a) benign and malignant prostate microsomes, (b) Hep G2 hepatoma microsomes, and (c) MDA-MB-231 microsomes by placental NADPH cytochrome *c* (*P*-450) reductase IgG.

tumors is higher than that in the surrounding tissues [21, 22]. Our own unpublished data suggest that aromatase activity in malignant prostate is higher than that of the benign tissue, although it must be stated that it is as yet uncertain whether the ^3H_2O released in the assay used for measurement of aromatase activity is actually produced by aromatase itself or 5α reductase [23]. It is possible that the increase in aromatase activity in neoplastic tissue is initiated via the NADPH cytochrome *c* (*P*-450) reductase component of the cytochrome *P*-450 enzyme system responsible for oestrogen biosynthesis rather than by a direct effect on cytochrome *P*-450 aromatase and its genetic machinery by carcinogens and growth factors [24]. The MDA cells were cultured from pleural fluid infusion from a patient with breast cancer and are therefore the result of tumour metastasis. The results indicate that the NADPH cytochrome *c* reductase activity of the MDA microsomes were significantly lower ($P < 0.05$) than that of breast adipose microsomes. Immunochemically the MDA microsomes showed 6% less homology towards the placental antibody than did the breast adipose preparation. These results may suggest that some kind of transformation has occurred during neoplasia. The results for benign and malignant prostate showed little evidence of any immunochemical or catalytic changes that might have occurred during the progression of the neoplastic process in this tissue.

In conclusion our results demonstrate that human placental NADPH cytochrome *c* (*P*-450) reductase shares common antigenic structures necessary for activity with that of the enzyme in the following tissues: breast adipose, prostate (benign and malignant), Hep G2 hepatoma cells and MDA cells. Conclusive evidence of enzyme structural or conformational changes as a result of neoplasia relevant to NADPH cytochrome *c* reductase activity was not obtained. Although polyclonal antibodies have been successful in detecting protein structure and conformation changes, their lack of specificity may have been a significant disadvantage in this type of study. The use of monoclonal antibodies with their capability of recognizing single epitopes [25] would probably constitute a more valuable probe in recognizing changes that might occur in tissues consequent to the onset and progression of neoplasia.

REFERENCES

1. Vermilion J. L., Ballou D. P., Massey V. and Coon M. J.: Separate roles for FMN and FAD in catalysis by liver microsomal NADPH-cytochrome *P*-450 reductase. *J. Biol. Chem.* **256** (1981) 266–277.
2. Conney A. H.: Pharmacological implications of microsomal enzyme induction. *Pharmac. Rev.* **19** (1967) 317–366.
3. Guengerich F. P.: Roles of cytochrome *P*-450 enzymes in chemical carcinogenesis and cancer chemotherapy. *Cancer Res.* **48** (1988) 2946–2954.
4. Hall P. F.: Role of cytochromes *P*-450 in the biosynthesis of steroid hormones. *Vit. Horm.* **42** (1985) 315–368.
5. Guengerich F. P.: Polymorphism of cytochrome *P*-450 in humans. *Trends Pharmac. Sci.* **10** (1989) 107–109.
6. Hiwatushi A. and Ichikawa T.: Purification and organ specific properties of cholecalciferol 25 hydroxylase system: cytochrome $P\text{-}450_{D25}$ linked mixed function oxidase system. *Biochem. Biophys. Res. Commun.* **97** (1980) 1443–1449.
7. Osawa Y., Higashiyama T. and Nakamura T.: Species specificity of estrogen biosynthesis in pregnancy. Immunochemical difference of placental NADPH cytochrome *c* (*P*-450) reductase in human, baboon and horse. *J. Steroid Biochem.* **15** (1981) 449–452.
8. Guengerich F. P., Wang P. and Mason P. S.: Immunological comparison of rat, rabbit and human liver NADPH cytochrome *P*-450 reductases. *Biochemistry* **20** (1981) 2379–2385.
9. Guengerich F. P. and Martin M. U.: Purification of cytochrome *P*-450, NADPH cytochrome *P*-450 reductase and epoxide hydrase from a single preparation of rat liver microsomes. *Archs Biochem. Biophys.* **205** (1980) 365–379.
10. Laemmli U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** (1970) 680–685.
11. Weir D. M.: *Handbook of Experimental Immunology*. Blackwell, Oxford (1967) pp. 22–23.
12. Strobel H. and Dignam J. D.: Purification and properties of NADPH cytochrome *P*-450 reductase. In *Methods in Enzymology* (Edited by S. Fleischer and L. Pocker). Academic Press, New York, Vol. 611, Part E (1980) pp. 91–102.
13. Newton C. J., Samuel D. L. and James V. H. T.: Aromatase activity and concentration of cortisol, progesterone and testosterone in breast and abdominal adipose tissue. *J. Steroid Biochem.* **24** (1986) 1033–1039.
14. Lowry C. H., Rosenberg N. J., Farr A. L. and Randall R. J.: Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.* **193** (1951) 265–275.
15. Black S. D. and Coon M. J.: Structural features of liver microsomal NADPH cytochrome *P*-450 reductase. *J. Biol. Chem.* **257** (1982) 5929–5938.
16. Gum J. R. and Strobel H. W.: Isolation of the membrane binding peptide of NADPH-cytochrome *P*-450 reductase. *J. Biol. Chem.* **256** (1981) 7478–7486.
17. Masters B. S. S., Prough R. A. and Kamin H.: Properties of the stable aerobic and anaerobic half-reduced states of NADPH cytochrome *c* reductase. *Biochemistry* **14** (1975) 607–613.
18. Haniu M., McManus M. E., Birkett D. J., Lee T. D. and Shively J. E.: Structural and functional analysis of NADPH cytochrome *P*-450 reductase from human liver: complete sequence of human enzyme and NADPH-binding sites. *Biochemistry* **28** (1989) 8639–8645.
19. Bellino L.: Estrogen synthetase. Demonstration that the high molecular weight form of NADPH-cytochrome *c* reductase from human placental microsomes

is required for androgen aromatization. *J. Steroid Biochem.* **17** (1982) 261–270.
20. Wu H.Q., Brown J. M., Tweedie D. J., Milewich L., Frankel R. A., Wixtrom C. M., Estabrook R. W. and Prough R. A.: Induction of microsomal NADPH-cytochrome *P*-450 reductase and cytochrome *P*-450 IV AI (P-$450_{LA\omega}$) by dehydroepiandrosterone in rats: a possible peroxisomal proliferator. *Cancer Res.* **49** (1989) 2337–2343.
21. James V. H. T., McNeill J. M., Lai L. C., Newton C. J., Ghilchik M. W. and Reed M. J.: Aromatase activity in normal breast and breast tumor tissues: *in vivo* and *in vitro* studies. *Steroids* **50** (1987) 269–279.
22. O'Neill J. S., Elton R. A. and Miller W. R.: Aromatase activity in adipose tissue from breast quadrants: a link with tumour site. *Br. Med. J.* **296** (1988) 741–743.
23. Brodie A. M. H., Son C., King D. A., Meyer K. M. and Inkster S. E.: Lack of evidence for aromatase in human prostatic tissues: effects of 4-hydroxyandrostenedione and other inhibitors on androgen metabolism. *Cancer Res.* **49** (1989) 6551–6555.
24. Lippman M. E., Dickson R. B., Bates S., Knabbe C., Huff K., Swain S., McManaway M., Bronzert D., Kasid A. and Gelmann E. P.: Autocrine and paracrine growth regulation of human breast cancer. *Breast Cancer Res. Treat.* **7** (1986) 59–70.
25. Katzir E. K. and Kenett D.: Use of monoclonal antibodies in the study of the conformation and conformational alterations in proteins. *Bull. Chem. Soc. Jap.* **61** (1988) 133–139.