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Immunohistochemical study of cytochrome P450 2C and 3A in human non-neoplastic and neoplastic tissues

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Abstract Organ and cellular distribution and expression constancy of microsomal cytochrome P450 (CYP) 2C and 3A in humans were studied with new polyclonal antibodies to CYP2C (MP-1) and 3A (NF-2) active in formalin-fixed, paraffin-embedded tissues. Antibodies were raised against purified human CYP2C9 and CYP3A4. On western blotting, MP-1 reacted with 2C8, 2C9, 2C18 and 2C19, and NF-2 with 3A4. In both frozen and paraffin sections, hepatocytes showed diffuse immunoreactivity with MP-1 and centrilobular staining with NF-2. In paraffin sections of 40 kinds of nonneoplastic tissues, epithelium of the small and large intestine, bile duct, nasal mucosa, kidney and adrenal cortex stained positively with both MP-1 and NF-2 antibodies. Epithelium of gastric fundic glands, salivary glands, tracheobronchial glands, Brunner's glands, the prostate, uterine cervix and nasopharynx showed definite reactivity with MP-1. Epithelium of the gastric mucosa with intestinal metaplasia, duodenum, gallbladder and intercalated ducts of the pancreas and chief cells of the parathyroid and the corpus luteum of the ovary reacted with NF-2. Among the neoplastic tissues, MP-1 reacted with pleomorphic adenoma of the salivary gland and carcinomas of six different organs, and NF-2 with those of 7 different organs. These results indicate that CYP2C and CYP3A are distributed widely and organ specifically, as well as being variably expressed in neoplastic and normal states.

Key words Human · Cytochrome P450 2C · Cytochrome P450 3A · Immunohistochemistry · Neoplasms

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Introduction

Cytochrome P450 enzymes (CYPs) are important in the oxidative, peroxidative and reductive metabolism of endogenous and exogenous materials. They metabolize a wide range of foreign chemicals, including drugs, environmental pollutants, natural plant products and alcohols. CYPs function not only in the detoxification of internal and external xenobiotics but also in the metabolic activation of carcinogens and may be involved in tumour initiation, promotion and progression [1]. Degawa et al. reported that DNA adducts detected in the human larynx were largely derived from metabolic activation of polycyclic aromatic hydrocarbons in cigarette smoke by P450 2C, 3A4, and/or 1A1 [2]. Moreover, specific CYPs have recently been shown to be involved in the metabolism of several essential anticancer agents [3]. Therefore, it is necessary to study the location, regulation, function and degradation of CYPs to elucidate their carcinogenic effects and develop methods of preventing or treating tumours.

CYP is most abundant in the liver, and CYP2C and 3A are major components of human liver microsomes [4]. The CYP3A subfamily constitutes about 25% of the total CYP present in liver microsomes [5]. These enzymes have an important role in liver detoxification. It is well known that CYP2C metabolizes tolbutamide, hexobarbital and warfarin and that CYP3A4 metabolizes testosterone, cortisol and aflatoxin B1 [6]. There is increasing interest in human CYP of extrahepatic organs, but in humans the evidence for associations of cancer risk with CYP composition or distribution is less well documented than in experimental animals [7].

CYPs have important roles in chemical carcinogenesis and the effects of cancer therapy, and immense interest has been focused on human CYP [8]. Most previous studies of CYP protein have addressed either the enzymatic activity or the mRNA level, because there are few human-specific antibodies that are usable in an immunohistochemical study. Anti-murine CYP antibodies have often been used in the study of human CYPs, but their specificity is questionable [9] and the recent development of molecular biology has demonstrated the enormous differences between human and rodent genes and their product structures [10]. Until recently, the lack of availability of antibodies with sufficient specificity has precluded immunohistochemical studies of human CYP2C and 3A. In addition, the formalin fixation used for surgical pathology renders the antigens undetectable [11]. For systemic studies of CYP distribution, it is important to generate antibodies capable of detecting CYP2C and 3A in formalin-fixed, paraffin-embedded human tissues, which are the most readily available materials. With such antibodies, the immunohistochemical method offers advantages for detecting CYPs not only in organs but also at the cellular level and may provide information on their functions.

We report the generation of new polyclonal antibodies against purified human CYP2C and 3A, which can be applied to formalin-fixed, paraffin-embedded tissues. Using these antibodies against CYP2C and 3A, we studied their distribution and frequency of expression in hepatic and extrahepatic organs in nonneoplastic and neoplastic states.

Materials and methods

CYP2C9 and CYP3A4 were purified from liver microsomes of a human autopsy sample by previously described methods with slight modifications [12, 13]. Liver microsomes were solubilized with 1.0% cholic acid (w/v) in 100 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol (v/v), 1.0 mM EDTA, and 1.0 mM dithiothreitol. The solubilized supernatant was subjected to *n*-octylamino–Sepharose 4B column chromatography as described previously [12]. The main CYP fraction was recovered from the column with 100 mM potassium phosphate buffer containing 0.40% cholic acid (w/v) and 0.06% Emulgen 911 (w/v). The peak CYP fraction thus obtained was dialysed against 20% glycerol (v/v) containing 0.1 mM dithiothreitol to lower the phosphate concentration and passed through a hydroxy apatite column, which had been equilibrated with 40 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol (v/v), 0.1 mM dithiothreitol, and 0.20% Emulgen 911 (w/v). The elution of CYP from the column was achieved by increasing the phosphate concentration from 40 mM to 300 mM (linear gradient, total volume of 600 ml), and three peak fractions with CYP activities were obtained. The peak II fraction contained CYP proteins immunoreactive to an anti-CYP3A4 antibody (Gentest Corporation, Woburn, Mass.), and the peak III fraction contained protein immunoreactive with anti-CYP2C9 antibodies (Gentest Corporation). The peak II fraction was applied to a CM-52 CM-cellulose column (1.6×1.6 cm, Whatman Japan, Tokyo, Japan), which had been equilibrated with 7.5 mM potassium phosphate buffer (pH 6.75) containing 20% glycerol (v/v), 0.2 mM dithiothreitol, 0.1 mM EDTA, and 0.20% Emulgen 911 (w/v), and the early fractions were found to yield a highly purified preparation of CYP3A4. CYP2C9 was further purified from the peak fraction III of the hydroxy apatite column onto DE-52 and CM-52 column chromatographies as described previously [12]. Detergent was removed from the purified CYP enzymes in a small column of hydroxy apatite.

Analysis of the first 25 N-terminal amino acid sequences of two purified preparations suggested that they matched the predicted gene sequences of CYP2C9 and CYP3A4 [1], respectively, except that the first methionine in the CYP3A4 preparation was missing.

Antibodies were raised against CYP2C and CYP3A in three Japanese White rabbits (Charles River Japan, Yokohama, Japan) according to the sensitization schedule described previously [14]. IgG of each immunized animal was prepared from the serum with an Ampure TM PA kit (Amersham Japan, Tokyo, Japan).

The specificity of anti-CYP2C IgG (MP-1) was assessed by its effects on the metabolism of diclofenac, mefenamic acid, *S*-mephenytoin and tolbutamides in human liver microsomes. The specificity of anti-CYP3A IgG (NF-2) was assessed by its effect on the metabolism of telosterone in human liver microsomes. NF-2 inhibited testosterone 6-beta-hydroxylation by up to 90% in human liver microsomes (unpublished data).

The normal and the neoplastic tissues used in this study are listed in Tables 1 and 2, respectively. The formalin-fixed, paraffin-embedded tissues were selected from the files of the Pathology Division, Tsukuba Medical Center, Ibaraki, Japan, and the Pathology Division, National Cancer Center Hospital East, Chiba, Japan. At least six neoplastic tissue samples were chosen, to include a variety of morphological differentiation, namely well-, moderately and poorly differentiated types, and grades (high, intermediate and low). Nonneoplastic tissues were separated from pathologic material. Tissues had been fixed in 10% neutral buffered formalin and embedded in paraffin wax. Fresh liver tissues were obtained from autopsy cases and frozen as samples not only for western blotting but also for immunohistochemistry, to compare the immunoreactivities of frozen and formalin-fixed, paraffin-embedded tissues.

For microsome preparation and western blotting, frozen liver was rinsed in ice-cold 0.9% NaCl solution. The tissue was minced with scissors and homogenized in 10 mM Tris-HCl. Microsomes were prepared from the homogenates by differential centrifugation [15]. Microsomal pellets were suspended with phosphate buffer 100 mM, pH 7.4 containing 20% glycerol and 10 mM $MgCl_2$. Microsomal proteins (50 mg) were separated by electrophoresis on sodium dodecyl sulfate polyacrylamide gels containing 10% acrylamide, as described by Laemmli [16]. Resolved proteins were electrotransferred to nitrocellulose membranes, which were probed with the antibodies and stained as described previously [17]. In order to specify the subfamily detected by the antibodies, we used 50 ml of microsomal protein solution containing CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1 or 3A4 (Gentest). The CYP concentrations of these solutions were 0.5, 1.1, 1.1, 1.6, 0.3, 0.5, 0.1, 0.4, 2.0 and 0.7 nM/ml, respectively. We also used control microsomes without CYP (Gentest).

Sections 4 mm thick cut from each paraffin block were attached to 3-aminopropyltriethoxysilane-coated slides (Muto, Tokyo, Japan) for immunohistochemistry. The sections were dewaxed by passage through xylene and hydrated with graded ethanols. The deparaffinized sections were washed three times in phosphate-buffered saline (PBS) for 10 min and immersed in methyl alcohol containing 3% H_2O_2 for 30 min to minimize the endogenous peroxidase activity. After another rinse in PBS, normal swine serum was applied and the sections were incubated for 30 min at room temperature in a humidified chamber. The sections were then incubated overnight with primary antibody MP-1 or NF-2 diluted at 1:2000, followed by biotinylated anti-rabbit IgG (1:300 dilution; Dako, Copenhagen, Denmark) and streptoavidin-biotinylated horseradish peroxidase complex (1:300 dilution, Dako) for 30 min each. The colour reaction was developed with 0.05% 3,3´-diaminobenzidine-tetrahydrochloride (Dojindo, Kumamoto, Japan) and 0.005% hydrogen peroxide in 0.05 M Tris solution (pH 7.6). Between incubations, the sections were always washed three times in PBS. After colour development, the sections were counterstained with haematoxylin. Serial sections were used as a negative control study by substituting normal rabbit serum or PBS for the primary antibodies.

Table 1 Immunohistochemical localizations of CYP2C and 3A in normal human tissues using polyclonal antibodies, MP-1 and NF-2 (*C* constant expression, *F* frequent expression, *O* occasional expression, –, no expression, *d* diffuse distribution, *f* focal distribution)

Tissue	CYP2C	CYP3A
Digestive system		
Tongue	–	–
Salivary glands	Intercalated duct epithelium (Cd) Serous gland (Cf)	–
Esophagus	Glandular epithelium (Of)	–
Stomach	Parietal cells of fundic gland (Fd)	Intestinal foveolar epithelium (Cd)
Duodenum	Brunner's gland epithelium (Od)	Surface epithelium (Cd)
Jejunum	Surface epithelium (Od)	Surface epithelium (Od)
Ileum	Surface epithelium (Od)	Surface epithelium (Od)
Appendix	–	–
Colon	Surface epithelium (Od)	Surface epithelium (Od)
Rectum	Surface epithelium (Od)	Surface epithelium (Od)
Liver	Hepatocytes (Cd)	Hepatocytes (Cf)
Galbladder	–	Surface epithelium (Of)
Bile duct	Surface epithelium (Of)	Surface epithelium (Of)
Pancreas	Islet cell (Cf)	Intercalated duct epithelium (Ff)
Respiratory system		
Nasopharynx	Ciliated and glandular epithelium (Ff)	–
Nasal mucosa	Serous cells of glands (Cd)	Serous cells of glands (Cf)
Trachea	Serous cells of glands (Cd)	–
Lung	Serous cells of bronchial glands (Cd)	–
Urogenital system		
Kidney	Proximal tubules (Of)	Proximal tubules (Of)
Bladder	–	–
Testis	–	–
Prostate	Secretory epithelium (Ff)	–
Ovary	–	Granulosa lutein cells (Cf)
Uterus	Cervical epithelium (Ff)	–
Mammary gland	–	–
Placenta	–	–
Endocrine glands		
Thyroid	–	–
Parathyroid glands	–	Chief cells (Of)
Adrenal glands	Zona reticularis (Cd)	Zona glomerulosa (Cd)
Others		
Skin	–	–
Ganglia	–	–
Cerebral cortex	–	–
Cerebellum	–	–
Tonsil	–	–
Lymph nodes	–	–
Spleen	–	–
Bone marrow	Myeloid cells (Cd)	Plasma cells (Cd)
Inflammatory cells	Neutrophils, basophils (Cd)	Plasma cells (Cd)
Heart, Aorta	–	–

We evaluated the immunohistochemical results at four expression frequencies in the organs and at two positive ratio grades, as follows. Constant expression: every sample showed positive immunoreactivity; frequent expression: the majority of samples showed positive immunoreactivity; occasional expression: less than half the samples showed positive immunoreactivity; no expression: none of the samples showed positive immunoreactivity; diffuse reaction: more than 50% of cells in the same lineage showed immunoreactivity; focal reaction: less than 50% of cells from one lineage showed immunoreactivity.

Table 2 Immunohistochemical localizations of CYP2C and 3A in neoplastic human tissues using polyclonal antibodies, MP-1 and NF-2 (*No.* number of cases examined, *ca* carcinoma, *Adenoca* adenocarcinoma, *(a)* focal or scattered distribution)

Organs	Tumours	No.	CYP2C	CYP3A
Digestive system				
Tongue	Squamous cell ca	6	0	0
Esophagus	Squamous cell ca	6	0	0
Salivary glands	Pleomorphic adenoma	8	6	0
Stomach	Tubular adenoca	8	0	2
	Poorly differentiated adenoca	6	0	0
Colon	Tubular adenoma	6	0	1(a)
	Tubular adenoca	9	3(a)	5(a)
Rectum	Tubular adenoca	6	0	3(a)
Liver	Hepatocellular ca	8	2	4
Gallbladder	Tubular adenoca	6	0	0
Bile duct	Tubular adenoca	6	0	0
Pancreas	Tubular adenoca	6	0	0
Respiratory system				
Nasal mucosa	Inverted papilloma	6	0	0
Lung	Squamous cell ca	6	0	0
	Adenoca	6	0	0
	Small cell ca	6	0	0
	Large cell ca	6	0	0
Urogenital system				
Kidney	Renal cell ca	6	0	0
Bladder	Transitional cell ca	6	0	0
Prostate	Adenoca	6	5(a)	0
Ovary	Serous ca	6	0	0
	Mucinous ca	6	0	0
Uterus	Squamous cell ca	6	0	0
	Endometrioid ca	6	0	0
Mammary gland	Invasive ductal ca	6	2	0
Endocrine glands				
Thyroid	Papillary ca	6	0	0
Adrenal glands	Adenoma	6	0	0
Others				
Meninges	Meningioma	6	0	0
Brain	Astrocytoma	8	0	0
Lymph node	Malignant lymphoma	6	0	0

Results

MP-1 recognized a single band of CYP2C8, 2C9 and 2C19 at MW 56,000 (Fig. 1a). NF-2 recognized only a single band of CYP3A4 at MW 57,000 (Fig. 1b). The lane of the negative control microsome showed no band reactive with MP-1 and NF-2.

In 11 human liver microsome samples, MP-1 and NF-2 detected single bands at MW 56,000 and 57,000, respectively (Fig. 2).

In frozen sections of the normal liver, MP-1-positive hepatocytes showed a diffuse but sometimes periportal distribution in hepatic lobules, while NF-2-positive hepatocytes were consistently present in the centrilobular area (Fig. 3a, b). A positive reaction was observed only in the cytoplasm, and not in the cell membrane or nucleus. Paraffin sections of liver specimens showed the same distribution pattern as frozen sections (Fig. 3c, d).

MP-1 and NF-2 positive cells were found in many extrahepatic organs (Table 1). Positive cells were limited to epithelial and haematopoietic cells, and no immunoreactivity was observed in the mesenchymal cells of any organs. Two distinct cytoplasmic immunoreactivities were observed, diffuse and localized. The latter pattern was usually present on the luminal sides of cells, forming tubular or glandular structures. No reactivity was detected in either the cytoplasmic membrane or the nucleus.

The distribution of CYP2C in normal tissues was as follows. The epithelium of intercalated ducts and the serous cells of salivary glands showed constant and diffuse immunoreactivity, while neither acinar mucous cells nor the epithelium of larger ducts was immunoreactive. The

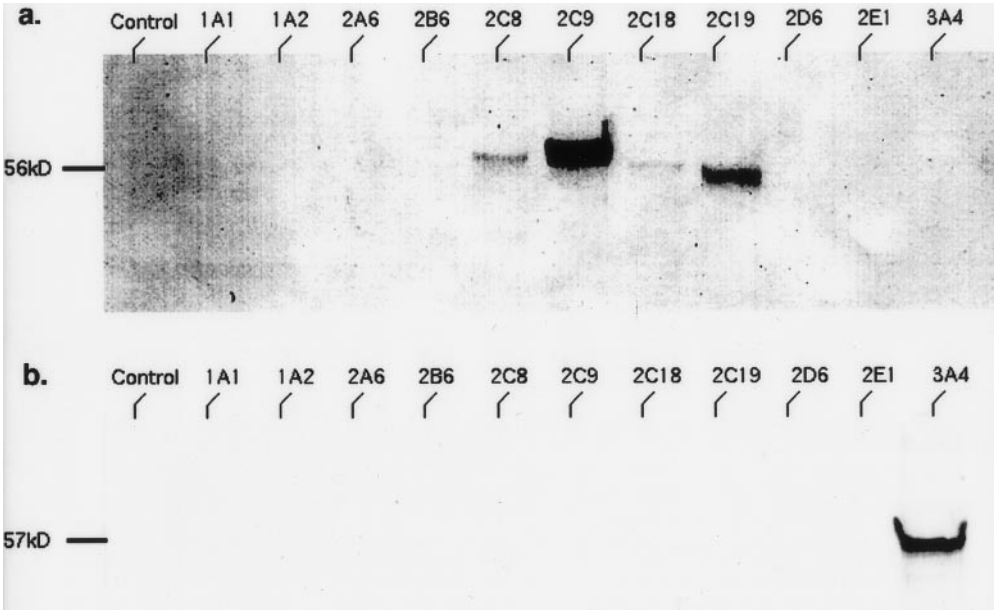


Fig. 1a, b Results of western blotting with MP-1 and NF-2, using control microsomes and microsomes containing several CYPs. **a** MP-1 recognizes a distinct band each of 2C9 and 2C19 and a faint band each of 2C8 and 2C18 at about MW 56,000. **b** NF-2 recognizes only a single band of CYP3A4 at MW 57,000 (*Control* negative control microsome)

chief cells of the glands of the gastric fundus showed constant and frequently diffuse immunoreactivity (Fig. 4a). The foveolar epithelium and mucous neck and parietal cells of the stomach were not immunoreactive. The surface epithelium of the small intestine and the columnar epithelium in the superficial portion of colonic and rectal crypts occasionally reacted with MP-1 (Fig. 4b). The middle portion of the crypts was weakly positive, while the bottom part was negative. Occasionally, we observed that Brunner's gland epithelium was diffusely immunoreactive (Fig. 4c), while the epithelium of the oesophageal gland and the surface epithelium of the bile duct showed focal immunoreactivity. Several immunoreactive islet cells were randomly distributed throughout the pancreas (Fig. 4d). There were no immunoreactive cells in the appendix or gallbladder.

We consistently observed immunoreactivity limited to the serous cells of nasal, nasopharyngeal, tracheal and bronchial glands (Fig. 4e). Only ciliated epithelium of the nasopharynx near the tonsil showed positive MP-1 immunoreactivity. Other ciliated and alveolar epithelia of the upper or lower respiratory tract were not immunostained.

Frequent but focal immunoreactivity was observed in the secretory gland epithelium of the prostate (Fig. 4f). The basal cells of the prostate were consistently negative. The glandular epithelium of the uterine cervix showed frequent but focal immunoreactivity (Fig. 4g). Metaplastic squamous cells in the uterine cervix were not immunostained. Occasionally, the proximal tubular epithelium of the kidney showed focal immunoreactivity. Glomeruli and other renal tubuli were uniformly negative. No immunoreactivity was detected in the urinary bladder, testis, ovary, breast or placenta.

In the adrenal glands, the cortical cells in the zona reticularis and the inner part of the zona fascicularis were diffusely immunoreactive. No immunoreactivity was detected in the adrenal medulla, thyroid or parathyroid.

Myeloid cells of the bone marrow and neutrophils within inflammatory lesions, regardless of the organs from which they were obtained, showed diffuse and constant immunoreactivity. Neural tissue including the cerebral cortex, cerebellum and ganglia and lymphoid cells from the tonsils, lymph nodes and spleen showed no immunoreactivity, and nor did the heart and aorta.

The distribution of CYP3A was also examined. The foveolar epithelium with intestinal metaplasia and duodenal epithelium showed constant and diffuse immunoreactivity (Fig. 5a). The nongoblet intestinal columnar cells were positive, while the goblet cells were consistently negative. No immunoreactivity was detected in pyloric or fundic gland mucosa, or in Brunner's glands of the duodenum. Columnar epithelium in the superficial part of the small intestine, and in colonic and rectal

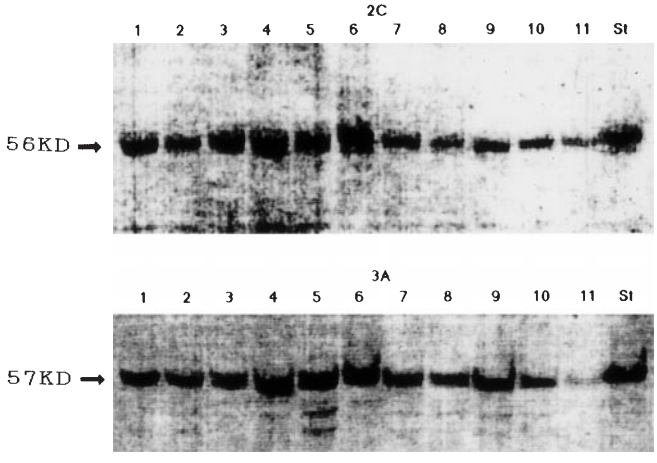


Fig. 2 Results of western blotting with MP-1 and NF-2, using 11 samples of human liver (*lanes 1–11*) and positive standard microsomes (*St*). MP-1 and NF-2 each recognize a single band, at MW 56,000 and 57,000, respectively

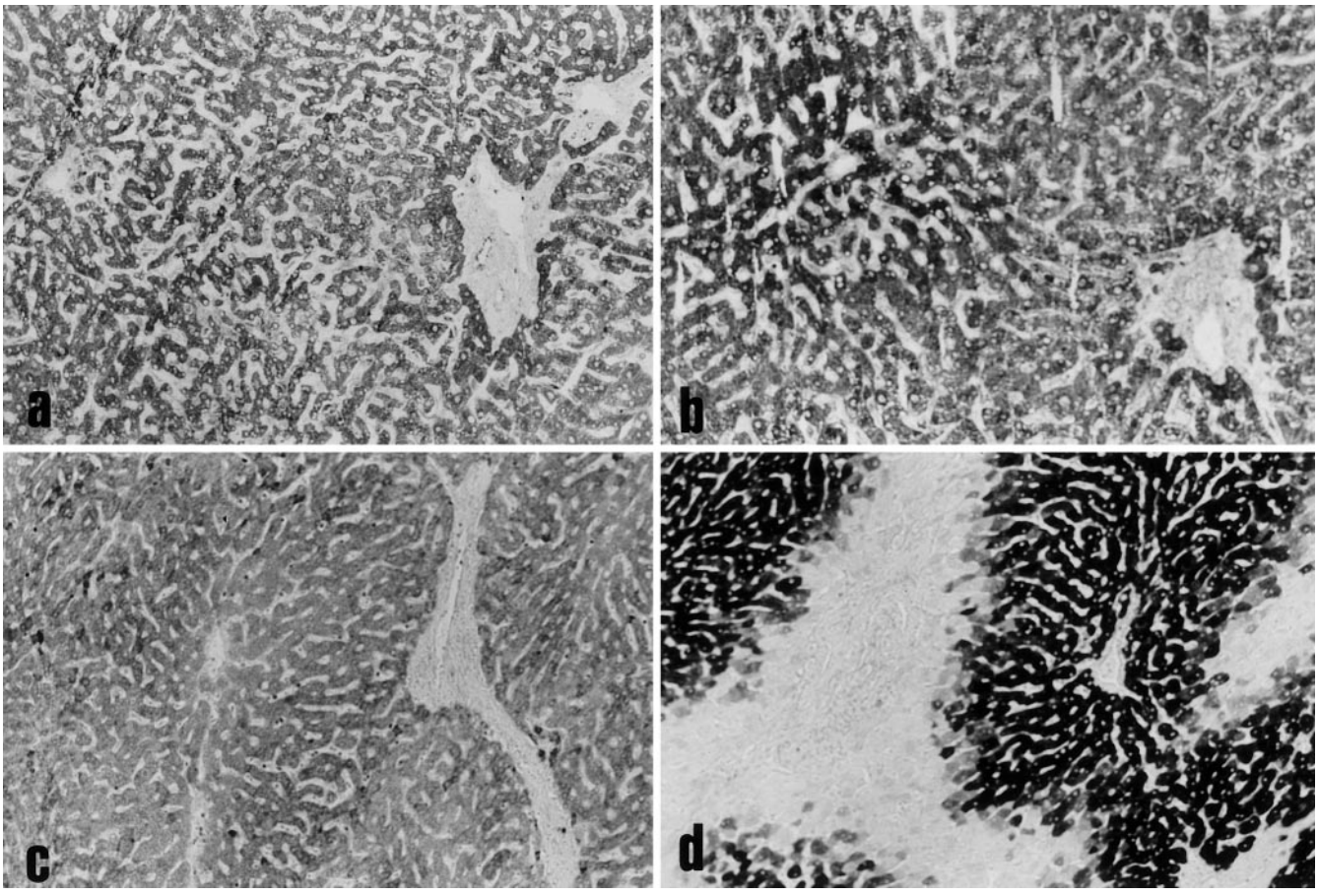


Fig. 3a–d Immunohistochemical localization of CYP using anti-CYP antibodies. In frozen sections of the liver, hepatocytes reacted **a** diffusely with MP-1 and **b** centrilobularly with NF-2. In paraffin sections of the liver, hepatocytes showed the same distribution of CYPs, based on **c** MP-1 and **d** NF-2 immunostaining, as frozen sections. ×120

crypts, reacted with NF-2 (Fig. 5b). The crypts were weakly reactive in the middle portion but showed no immunoreactivity at the bottom. The surface epithelium of the gallbladder and bile ducts occasionally showed focal immunoreactivity. The epithelium of the intercalated ducts in the pancreas was frequently positive (Fig. 5c), while that of the salivary glands was negative.

Serous glands of the nasal mucosa showed consistent but focal immunoreactivity (Fig. 5d). There were no positive cells in the nasopharynx, trachea or lungs.

The epithelium of the proximal renal tubules occasionally showed focal immunoreactivity (Fig. 5e). Distal and collecting tubuli were consistently negative. The cytoplasm of the luteinized granulosa cells was consistently but focally positive (Fig. 5f). The transitional cells of the urinary bladder, spermatogenic cells in the testis, ductal and acinar cells of the breast and trophoblasts in the placenta were negative. Squamous and glandular epithelium of the uterine cervix and epithelium of the endometrium in proliferative and secretory phases were also devoid of immunoreactivity.

Chief cells of the parathyroid gland showed weak but diffuse immunoreactivity (Fig. 5g). The zona glomerulosa of the adrenal gland was consistently, diffusely immunoreactive (Fig. 5h).

Nervous tissue including ganglia, the cerebral cortex and the cerebellum, and lymphoid organs such as the tonsils, lymph nodes and spleen, were negative. Plasma cells showed consistent immunoreactivity in the bone marrow and inflammatory lesions.

Several neoplasms showed immunoreactivity with MP-1 and NF-2 (Table 2). MP-1 reacted with the glandular epithelium of pleomorphic adenoma of the parotid gland (6 of 8 cases). Hepatocellular carcinoma immunoreacted with MP-1 in 2 of 8 cases, both of them well-differentiated tumours (Fig. 6a). Tumour cells from adenocarcinoma of the colon (3 of 9 cases), adenocarcinoma of the prostate (5 of 6 cases) and infiltrating ductal carcinoma of the breast (2 of 6 cases; Fig. 6b) were also positive. No immunoreactivity was observed in any case of carcinoma of the tongue, oesophagus, stomach, gallbladder, bile duct, pancreas, lung, uterus, kidney, urinary bladder, ovary or thyroid, or of astrocytoma and meningioma of the brain or malignant lymphoma involving the lymph nodes. NF-2 showed widespread reactivity with hepatocellular carcinoma in 4 of 8 cases and the positive cases had well- to moderately differentiated tumours (Fig. 6c). Tumour cells from adenocarcinomas of the stomach (2 of 8 cases) and large intestine (8 of 15 cases, Fig. 6d) reacted with NF-2. How-

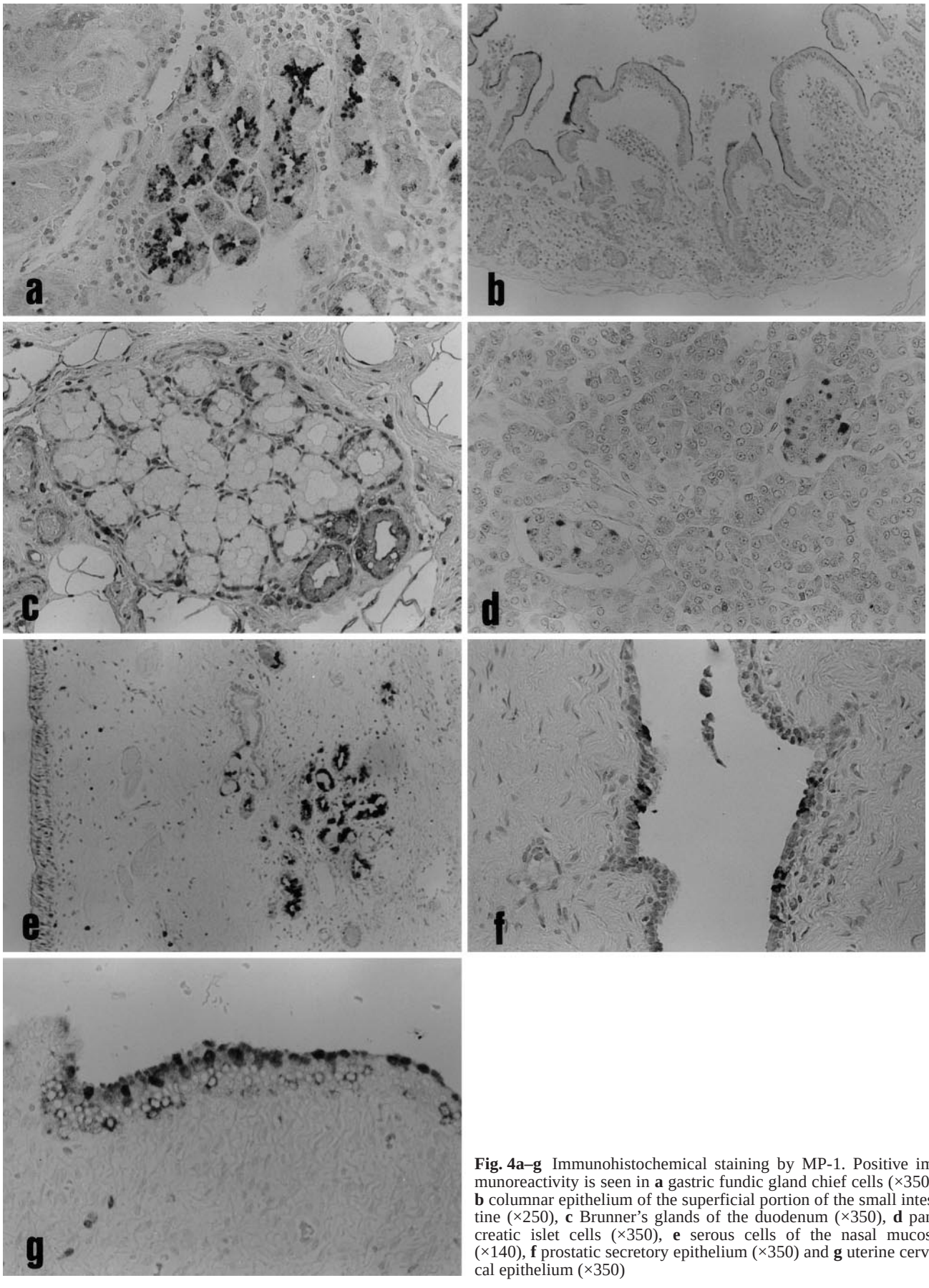
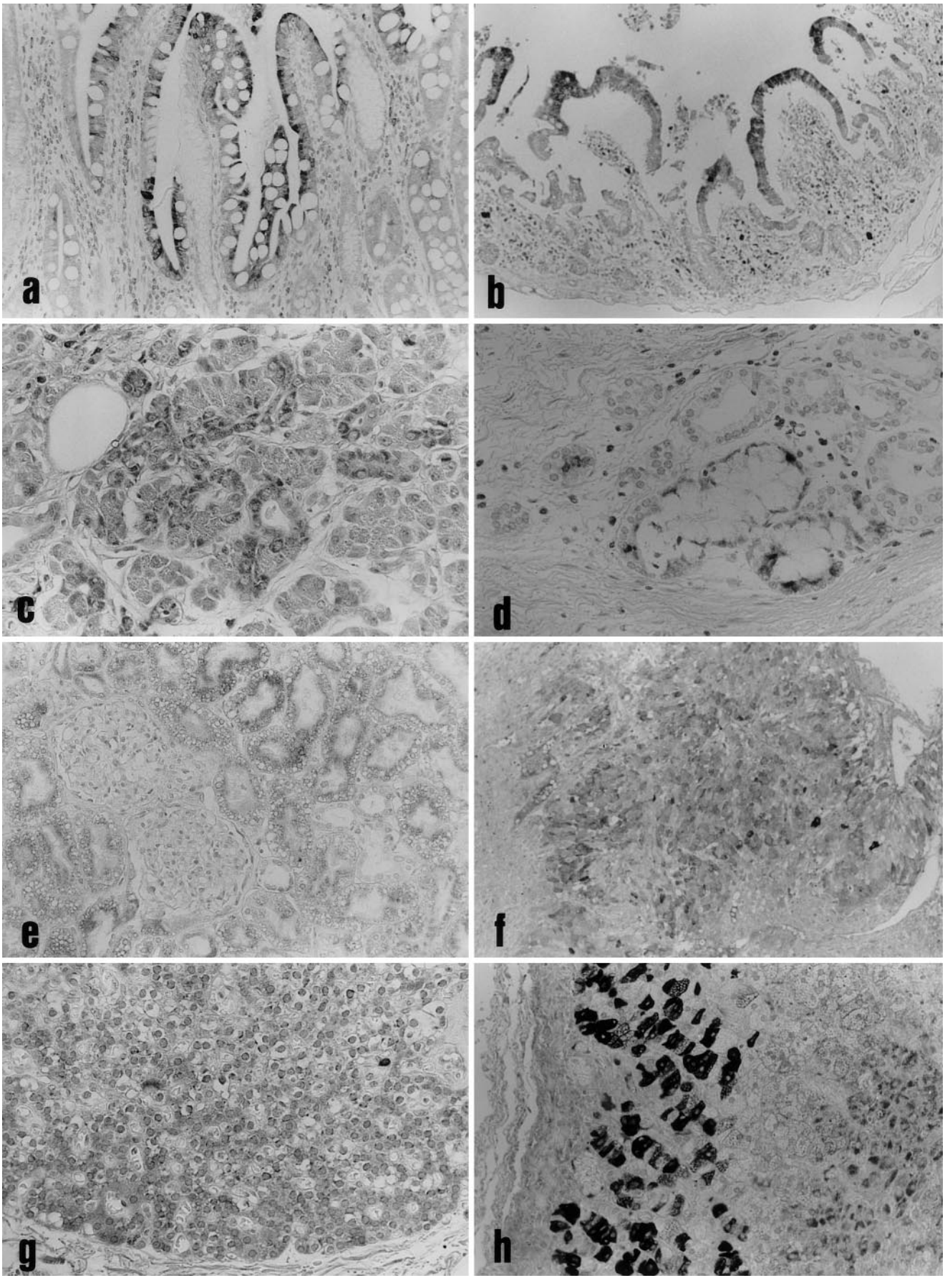


Fig. 4a–g Immunohistochemical staining by MP-1. Positive immunoreactivity is seen in **a** gastric fundic gland chief cells (×350), **b** columnar epithelium of the superficial portion of the small intestine (×250), **c** Brunner's glands of the duodenum (×350), **d** pancreatic islet cells (×350), **e** serous cells of the nasal mucosa (×140), **f** prostatic secretory epithelium (×350) and **g** uterine cervical epithelium (×350)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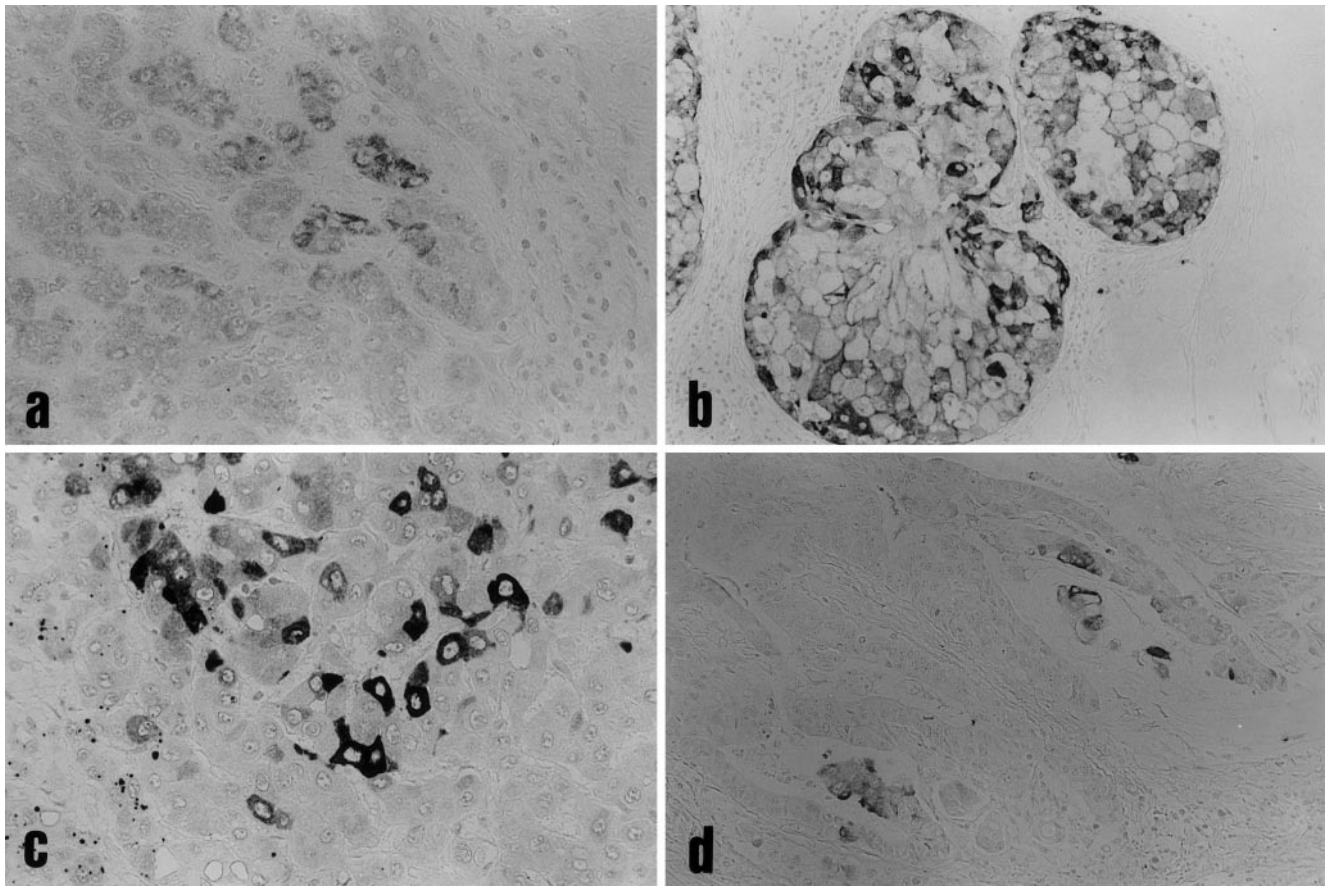

Fig. 6a–d Immunohistochemical detection of CYP in neoplastic tissues. MP-1 is **a** weakly immunoreactive with hepatocellular carcinoma (×360), but **b** shows strong positivity in an infiltrating ductal carcinoma of the breast (×180). NF-2 is immunoreactive with **c** hepatocellular carcinoma (×360) and **d** adenocarcinoma of the colon (×220)

ever, no immunoreactivity was observed in any case of pleomorphic adenoma of the parotid gland, carcinoma of the tongue, oesophagus, gallbladder, bile duct, pancreas, lung, ovary, uterus, kidney, urinary bladder, prostate, breast or thyroid, or of glioma of the brain and malignant lymphoma. In all the neoplastic tissues examined, immunoreactivity was limited to the cytoplasm, none being detected in the cytoplasmic membrane or nucleus.

Discussion

The results of western blotting showed that MP-1 and NF-2 were specific for CYP2C and CYP3A4. Using frozen and paraffin sections of the liver tissue, the cellular distribution of CYP2C detected with MP-1 was diffuse within the lobules, while CYP3A detected with NF-2 was distributed in the centrilobular area, in accordance with the findings of Murray et al. [18]. Since the frozen and paraffin sections of the liver showed the same localization pattern and intensity of CYPs with MP-1 and NF-2, we considered these antibodies could be used in an immunohistochemical study using formalin-fixed, paraffin-embedded tissues.

Few studies have demonstrate the extrahepatic expression of CYP2C immunohistochemically. CYP2C was reported to be present in the prostatic gland epithelium [19] and to be absent in the transitional epithelium of the urinary bladder and in normal squamous epithelium and squamous cell carcinoma of the oesophagus [20, 21]. The current study confirms that normal squamous epithelium of the oesophagus, tongue, nasal mucosa, skin, tonsil or uterine cervix does not express CYP2C. As it appears to be rare for normal squamous epithelium to express CYP2C, as demonstrated immunohistochemically, we speculate that cell differentiation may be related to the regulation of CYP expression; however, this possibility requires further study. No immunohistochemical studies have detected CYP2C expression in other organs; therefore, we can only compare the results with those of immunoblot analysis. De Waziers et al. reported that immunoblot analysis of gastrointestinal organs revealed CYP2C activity in the duodenum, jejunum and ileum, though the expression levels were very low [22]. The present obser-

Fig. 5a–h Immunohistochemical staining with NF-2. Positive immunoreactivity is seen in **a** foveolar epithelium of the stomach with intestinal metaplasia (×180), **b** luminal surface epithelium of the small intestine (×140), **c** intercalated duct epithelium of the pancreas (×360), **d** serous cells of the nasal mucosa (×330), **e** proximal tubules of the kidney (×200), **f** granulosa lutein cells of the ovary (×130), **g** chief cells of the parathyroid gland (×360) and **h** the zona glomerulosa of the adrenal gland (×250)

vation that the luminal side of the surface epithelium from the jejunum to the colon expresses CYP2C is consistent with the findings of de Waziers et al. The duodenum showed a different localization of CYP2C; CYP2C was expressed by the surface epithelium but not by Brunner's glands. Wheeler et al., using an immunoblotting method, reported that CYP2C9 was not detectable in microscomes from the lung [23]. However, the present study showed CYP2C to be expressed in the serous cells of bronchial glands. These cells are similar to serous cells in the nasal and bronchial mucosa. It is possible that Wheeler et al. used such samples as peripheral lung tissue, which may not have included bronchial gland tissues.

CYP3A is a major CYP in the liver. Thus, there are more reports on the extrahepatic localization of this enzyme than on that of CYP2C. Murray et al. reported the localization of CYP3A4 in 18 types of tissue using a monoclonal antibody, MAb-HL3 [18]. As in our study, they found that CYP3A4 was localized in the surface epithelium of the small intestine, the gallbladder epithelium, the proximal renal tubular epithelium, and lutein cells of the ovary. However, they also reported the expressions of CYP3A4 in the acini of the pancreas, Leydig cells of the testis, epidermis and sebaceous glands of the skin and polymorphonuclear cells in the peripheral blood, which were negative for CYP3A4 in our study. Moreover, we observed CYP3A4 in the foveolar epithelium of the stomach with intestinal metaplasia, the surface epithelium of the colon, serous cells of nasal glands and cells in the zona glomerulosa of the adrenal gland, where Murray et al. reported absence of this enzyme. The discrepancies may be attributable to the following three factors: (1) absence of positive tissue, e.g. foveolar epithelium of the stomach with intestinal metaplasia, (2) different antibody specificities and sensitivities of the immunohistochemical detection system [18], and (3) the degree of extrahepatic CYP expression [24].

We found CYP3A in the epithelium of the gastrointestinal tract from the stomach to the rectum, with the exception of the appendix. Our results confirm those of Kolars et al. [25]. We recognized marked expression in the foveolar epithelium of the stomach with intestinal metaplasia, but Kolars et al. did not refer to metaplastic epithelium. Because intestinal metaplasia has been postulated to be one of the steps in human gastric carcinogenesis and several carcinogens and/or carcinogenetic enhancers influence each step of the multistep process [26], the CYP3A associated with the intestinal metaplasia might be involved in the transformation from metaplastic epithelium to carcinoma.

Murray et al. reported that the colonic epithelium showed no immunostaining for CYP3A [18], while other workers have described CYP3A mRNA and proteins as detectable by in situ hybridization and immunoblotting [25, 27]. The current study demonstrated diffuse expression in CYP3A in the superficial portion of the colonic epithelium in some cases. These observations raise the possibility that the colonic mucosa interacts with xenobiotics, and that this reactivity varies among individuals. McKinnon et al. also demonstrated that CYP3A mRNA was expressed along the entire length of the human colon in some individuals, while there was no expression in the colon tissues from other subjects. They speculated that individual differences in CYP expression in the colon might be important in the metabolism of ingested procarcinogens, such as food-derived heterocyclic amines, and in susceptibility to cancer [28].

The present study has demonstrated the absence of CYP3A in the lung, the exception being the serous cells of pulmonary bronchial glands. Murray et al. also reported that the lung showed no immunoreactivity for CYP3A4 [18]. However, recently, some authors reported that there was CYP3A4 in the lung [3, 29, 30]. Kivistoe et al. reported that 34% of bronchial epithelium and 38% of seromucous gland cells were immunoreactive for CYP3A [3]. They also detected CYP3A5 mRNA, but not CYP3A4 mRNA, in the lung by the RT-PCR method. These findings suggest that the antibodies used both in the study of Murray et al. [18] and in the present investigation, are reactive with CYP3A4 but not with CYP3A5, while the antibody used by Kivistoe et al. reacts with CYP3A5. The different reactivities of the antibodies may explain the discrepant results. Alternatively, the method of Kivistoe et al. might have been sensitive enough to detect the lower expression of CYP3A in the lung, which expresses much less CYP3A than the human liver [23].

Our study demonstrated the presence of CYP2C and 3A in neoplastic cells from many organs. Nonneoplastic hepatocytes showed constant centrilobular expression of CYP2C, while hepatocellular carcinoma cells showed variable expression within a tumour and the immunoreactivity was decreased in less highly differentiated liver tumours. Similar trends were also noted in most other tumor types.

Breast cancer cells showed CYP2C immunoreactivity despite the negative result in the nonneoplastic breast tissue. Similar phenomena were also reported in the oesophagus and prostate and in sarcomas [19, 21, 31]. The expression of CYP in neoplastic cells may be a protective mechanism conferring a survival advantage by providing tumours with a metabolic mechanism for detoxification of harmful substances and drugs [32]. Therefore, immunohistochemical study of CYPs may provide information on the effectiveness of drugs against various tumours.

In conclusion, we studied the distributions of CYP2C and 3A in extrahepatic organs with newly generated antibodies and demonstrated the cellular distribution of these enzymes, some of which had been analysed only by non-immunostaining methods such as immunoblotting or genetic analysis. We also demonstrated the localization of CYP2C and 3A in various neoplasms. Extrahepatic CYP has been attracting interest in the field of pharmaco-oncology, because the balance between metabolic activation and detoxification of drugs and other chemicals by individual forms of CYP in different tissues is an important factor in the explanation of organ-specific toxicity and probably the efficacy of oncostatic drugs [33, 34]. The results of the present study provide the basic information that we anticipate will be useful for the pharmaco-oncological analysis of CYP in extrahepatic organs.

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Reference

1. Nelson DR, Kamataki T, Waxman DJ, Guengerich FP, Estabrook RW, Feyereisen R, Gonzalez FJ, Coon MJ, Gunsalus IC, Gotoh O, Okuda K, Nebert DW (1993) The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. DNA Cell Biol 12:1–51
2. Degawa M, Stern SJ, Martin MV, Guengerich FP, Fu PP, Ilett KF, Kaderlik RK, Kadlubar FF (1994) Metabolic activation and carcinogen-DNA adduct detection in human larynx. Cancer Res 54:4915–4919
3. Kivistoe KT, Fritz P, Linder A, Friedel G, Beaune P, Kroemer HK (1995) Immunohistochemical localization of cytochrome P450 3A in human pulmonary carcinomas and normal bronchial tissue. Histochemistry 103:25–29
4. Murray M, Reidy GF (1990) Selectivity in the inhibition of mammalian cytochrome P-450 by chemical agents. Pharmacol Rev 42:85–101
5. Watkins PB, Wrighton SA, Maurel P, Schetz EG, Mendez-Picon G, Parker GA, Guzelian PS (1985) Identification of an inducible form of cytochrome P-450 in human liver. Proc Natl Acad Sci USA 82:6310–6314
6. Miura SM, Mori M, Takiguchi M, Tatibana M, Furuta S, Miyazawa S, Hashimoto T (1984) Biosynthesis and intracellular transport of enzymes of peroxisomal beta-oxidation. J Biol Chem 259:6397–6402
7. Yuspa SH, Shields PG (1997) Etiology of cancer: chemical factors. In: DeVita VT, Hellman JS, Rosenberg SA (eds) Cancer: principles and practice of oncology, 5th edn. Lippincott-Raven, Philadelphia, pp 185–202
8. Guengerich FP (1988) Roles of cytochrome P-450 enzymes in chemical carcinogenesis and cancer chemotherapy. Cancer Res 48:2946–2954
9. Philip PA, Kaklamanis L, Ryley N, Stratford I, Wolf R, Harris A, Carmichael J (1994) Expression of xenobiotic-metabolizing enzymes by primary and secondary hepatic tumors in man. Int J Radiol Oncol Biol Phys 29:277–283
10. Kamataki T (1995) Molecular toxicology of cytochrome P450: focussing on interspecies homology (in Japanese). Yakugaku Zasshi 115:370–377
11. Van Pelt-Verkuil E, Eweis JJ (1981) The use of a carbodiimide-containing fixative for the immunohistochemical demonstration of coagulation factor VIII in cat vascular tissue. Histochemistry 71:187–194
12. Shimada T, Misono KS, Guengerich FP (1986) Human liver microsome cytochrome P-450 mephenitoin 4-hydroxylase, a prototype of genetic polymorphism in oxidative drug metabolism. Purification and characterization of two similar forms involved in the reaction. J Biol Chem 261:909–921
13. Mimura M, Baba T, Yamazaki Y, Ohmori S, Inui Y, Gonzalez FJ, Guengerich FP, Shimada T (1993) Characterization of cytochrome P450 2B6 in human liver microsomes. Drug Metab Dispos 21:1048–1056
14. Kaminsky LS, Fasco MJ, Guengerich FP (1981) Production and purification of antibodies to rat liver cytochrome P-450. Methods Enzymol 74:262–272
15. Kremers P, Beaune P, Cresteil T, De Graeve J, Columelli S, Leroux JP, Gielen JE (1981) Cytochrome P-450 monooxygenase activity in human and rat liver microsomes. Eur J Biochem 118:599–606
16. Laemmli UK (1970) Cleavage of structural properties during the assembly of the head of bacteriophage T4. Nature 227: 680–685
17. Beaune P, Flinois JP, Kiffel L, Kremers P, Leroux JP (1985) Purification of a new cytochrome P 450 from human liver microsomes. Biochim Biophys Acta 860:364–370
18. Murray GI, Barnes TS, Sewell HF, Ewen SWB, Melvin WT, Burke MD (1988) The immunohistochemical localization and distribution of cytochrome P-450 in normal human hepatic and extrahepatic tissues with monoclonal antibody to human cytochrome P-450. Br J Clin Pharmacol 25:465–475
19. Murray GI, Taylor VE, McKay JA, Weaver RJ, Ewen SW, Melvin WT, Burke MD (1995) The immunohistochemical localization of drug-metabolizing enzymes in prostatic cancer. J Pathol 177:147–152
20. Murray GI, Taylor VE, McKay JA, Weaver RJ, Ewen SW, Melvin WT, Murke MD (1995) Expression of xenobiotic metabolizing enzymes in tumours of the urinary bladder. Int J Exp Pathol 76:271–276
21. Murray GI, Shaw D, Weaver RJ, McKay JA, Ewen SW, Melvin WT, Burke MD (1994) Cytochrome P450 expression in oesophageal cancer. Gut 35:599–603
22. de Waziers I, Cugnenc PH, Yang CS, Leroux J-P, Beaune PH (1990) Cytochrome P450 isoenzymes, epoxide hydrolase and glutathion transferases in rat and human hepatic and extrahepatic tissues. J Pharmacol Exp Ther 253:387–394
23. Wheeler CW, Wrighton SA, Guenthner TM (1992) Detection of human lung cytochromes P450 that are immunochemically related to cytochrome P450IIE1 and cytochrome P450IIIA. Biochem Pharmacol 44:183–187
24. Kikkawa F, Nawa A, Oguchi H, Kawai M, Maeda O, Suganuma N, Aoyama T, Tomoda Y (1994) Positive correlation between cytochrome P450 2E1 mRNA level and serum estradiol level in human uterine endometrium. Oncology 51:52–58
25. Kolars JC, Lown KS, Schmiedlin-Ren P, Ghosh M, Fang C, Wrighton SA, Merion RM, Watkins PB (1994) *CYP3A* gene expression in human gut epithelium. Phamacogenetics 4:247–259
26. Correa P (1992) Human gastric carcinogenesis: a multistep and multifunctional process. Cancer Res 52:6735–6740
27. McKinnon RA, Burgess WM, Hall PM, Roberts-Thomson SJ, Gonzalez FJ, McManus ME (1995) Characterization of *CYP3A* gene subfamily expression in human gastrointestinal tissues. Gut 36:259–267
28. McKinnon RA, Burgess MW, Gonzalez FJ, McManus ME (1993) Metabolic differences in colonic mucosal cells. Mutat Res 290:27–33
29. Antilla S, Hukkanen J, Hakkola J, Stjernvall T, Beaune P, Edwards RJ, Boobis AR, Pelkonen O, Raunio H (1997) Expression and localization of CYP3A4 and CYP3A5 in human lung. Am J Resp Cell Mol Biol 16:242–249
30. Shimada T, Yamazaki H, Mimura M, Wakamiya N, Ueng YF, Guengerich FP, Inui Y (1996) Characterization of microsomal cytochrome P450 enzymes involved in the oxidation of xenobiotic chemicals in human fetal liver and adult lungs. Drug Metab Disp 24:515–522
31. Murray GI, Weaver RJ, Paterson PJ, Ewen SWB, Melvin WT, Burke MD (1993) Expression of xenobiotic metabolizing enzymes in breast cancer. J Pathol 169:347–353
32. Young RC (1989) Drug resistance: the clinical problem. In: Ozols R (ed) Drug resistance in cancer therapy. Kluwer Academic, Boston, pp 1–12
33. Gram TE, Okine LK, Gram RA (1986) The metabolism of xenobiotics by certain extrahepatic organs and its relation to toxicity. Annu Rev Pharmacol Toxicol 26:259–292
34. Krishna DR, Klotz U (1994) Extrahepatic metabolism of drugs in humans. Clin Pharmacokinet 26:144–160