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Tumor-specific Expression of Cytochrome P450 CYP1B1[1]

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ABSTRACT

Cytochrome P450 CYP1B1 is a recently cloned dioxin-inducible form of the cytochrome P450 family of xenobiotic metabolizing enzymes. An antibody raised against a peptide specific for CYP1B1 was found to recognize CYP1B1 expressed in human lymphoblastoid cells but not to recognize other forms of cytochrome P450, particularly CYP1A1 and CYP1A2. Using this antibody, the cellular distribution and localization of CYP1B1 were investigated by immunohistochemistry in a range of malignant tumors and corresponding normal tissues. CYP1B1 was found to be expressed at a high frequency in a wide range of human cancers of different histogenetic types, including cancers of the breast, colon, lung, esophagus, skin, lymph node, brain, and testis. There was no detectable immunostaining for CYP1B1 in normal tissues. These results provide the basis for the development of novel methods of cancer diagnosis based on the identification of CYP1B1 in tumor cells and the development of anticancer drugs that are selectively activated in tumors by CYP1B1.

INTRODUCTION

Cytochrome P450s are a multigene family of constitutive and inducible enzymes that have a central role in the oxidative metabolic activation and detoxification of both a wide range of xenobiotics (1–5) and several groups of endogenous compounds that are active in cell regulation and cell signaling, including arachidonic acid (6), steroid hormones (7), and fatty acids (8). The major families of cytochrome P450 involved in xenobiotic metabolism each consist of several individual forms with different regulatory mechanisms and substrate specificities (1). The majority of cytochrome P450s are primarily expressed in the liver (1, 4), although individual cytochrome P450 forms are also expressed in specific extrahepatic tissues, including small intestine, kidney, and lung (9–11).

The human CYP1 (individual cytochrome P450 forms are identified by the prefix CYP in accordance with the current cytochrome P450 nomenclature as described in Ref. 2) gene family, which is one of the major cytochrome P450 families involved in the metabolism of xenobiotics, is now known to consist of three individual forms classified into two subfamilies. The CYP1A subfamily contains two highly homologous and well-characterized but distinct members, CYP1A1 (12) and CYP1A2 (13). CYP1A1 is an inducible cytochrome P450 expressed primarily in extrahepatic tissues (14), whereas CYP1A2 is a major form of cytochrome P450 that is expressed constitutively in liver (15). Recently, a second human CYP1 subfamily has been identified, which to date contains one member, CYP1B1 (16). This cytochrome P450 is dioxin inducible, and sequence analysis of CYP1B1 shows 40% homology with both CYP1A1 and CYP1A2. Orthologous CYP1B1 forms have also been cloned recently from mouse (17) and rat (18, 19).

Several forms of cytochrome P450 are considered to have an important role in tumor development because they can metabolize many potential carcinogens and mutagens (20–23). Moreover, cytochrome P450 activity may influence the response of established tumors to anticancer drugs because several cancer chemotherapeutic agents can be either activated or detoxified by this enzyme system (22, 24). The presence of individual forms of cytochrome P450 has previously been investigated in different types of cancer, including breast (25), lung (26, 27), colon (28–31), and head and neck (32) cancer to determine whether intratumor metabolism of anticancer agents by cytochrome P450 could occur and thus influence the response of tumors to these agents. These studies have generally shown that the level of the cytochrome P450 forms investigated is significantly reduced or absent in tumors when compared with the adjacent normal tissue in which the tumors have developed. However, other studies, including studies in our laboratories, of several different types of cancer, including breast cancer (33), esophageal cancer (34, 35), and soft tissue sarcomas (36), have shown that there is expression of a CYP1 form of cytochrome P450 in these tumors. We have recently found that CYP1B1 mRNA is the most frequently expressed form of the CYP1 family mRNA in breast cancer (37), suggesting strongly that CYP1B1 is the main form of the CYP1 family present in breast cancer (37). In this study, we show that CYP1B1 shows enhanced expression in a wide range of malignant tumors and is not detectable in normal tissues.

MATERIALS AND METHODS

Tissue Samples. Samples of cancer and normal tissue from a wide range of tissues, including bladder, breast, colon, esophagus, kidney, lung, ovary, skin, stomach, uterus, connective tissue, lymph node, brain, and testis, were obtained from tissue specimens that had been submitted for diagnosis to the Department of Pathology, University of Aberdeen. These samples were from patients undergoing surgery for different types of malignant disease. For all tissues except lymph node, paired samples of normal and tumor tissues were available from each specimen of tissue. Normal lymph nodes were obtained from tissue samples removed for nontumor reasons. Normal liver and small intestine were also obtained with permission from organ transplant donors. For immunohistochemistry, the tissue samples had all been fixed in 10% neutral buffered formalin for 24 h at room temperature, and then blocks of both macroscopically normal and macroscopically tumor tissue were selected for histopathological analysis. These tissue blocks were then embedded in wax, and one section from each block was stained with H&E. These sections were examined by light microscopy, and histopathological diagnosis was made using established histological criteria. Samples of normal liver and breast cancer were also obtained fresh (not fixed in formalin), and these samples were used to prepare microsomes as described previously (38).

Anti-CYP1B1 Antibody. A new, specific, anti-CYP1B1 antibody was produced using a synthetic peptide corresponding to amino acids 332–345 of the deduced CYP1B1 amino acid sequence (39). This 14-amino acid peptide sequence was selected by comparing the CYP1B1 sequence with those of CYP1A1 and CYP1A2 and is specific for CYP1B1. A COOH-terminal cys-

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                          332          345
                           ↓            ↓
CYP1B1    APRDMMDAFILSAEKKAAGDSHGGGARLDLENVPATITDIFG
CYP1A1    I-RDITDSLIEHCQEKQL--DENANVQLSDEKIINIVLDLFG
CYP1A2    V-RDITGALFKH--SKKG--PRASGNLIPQEKIVNLVNDIFG
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Fig. 1. Amino acid sequence alignment of human CYP1B1, human CYP1A1, and human CYP1A2 showing the sequence (*underlined*) that was used for generation of the CYP1B1 antibody (*, amino acids common to all three cytochrome P450s).

teine was added for use in conjugation to keyhole limpet hemocyanin, and male New Zealand rabbits were immunized with the peptide conjugate. Each rabbit was immunized with 100 μg of peptide conjugate that was dissolved in 300 μl of PBS and mixed with 300 μl of Freund's complete adjuvant. The rabbits received further immunizations of 50 μg of peptide conjugate 3 and 4 weeks following the initial immunization. One week after the final immunization, collection of serum samples was commenced, and the serum was tested for CYP1B1 immunoreactivity. Those samples that showed a high titer were pooled, and the immunoglobulin fraction was obtained by ammonium sulfate precipitation.

The specificity of the antibody was tested by SDS-PAGE and immunoblotting (37, 40) with human liver microsomes, microsomes prepared from human breast cancer, and microsomes from human lymphoblastoid cells containing either expressed human CYP1B1 (Gentest Corp., Woburn, MA) or human CYP1A1 (Gentest). Proteins were electrophoretically separated at constant current using a 10% polyacrylamide gel and then transferred to nitrocellulose (Amersham International, Aylesbury, United Kingdom) by electroblotting (37). Nonspecific binding sites were blocked by incubation of the nitrocellulose membrane for 60 min in wash buffer consisting of 2% nonfat milk (Marvel, Premier Beverages, Stafford, United Kingdom) in 10 mM PBS containing 0.05% Tween 20 (Sigma Chemical Co., Poole, Dorset, United Kingdom). The nitrocellulose was then incubated sequentially with anti-CYP1B1 antibody (1:1000) and goat antirabbit immunoglobulin conjugated to horseradish peroxidase (1:2000; Bio-Rad, Hemel Hempstead, United Kingdom). After each antibody application, the membrane was washed for five 10-min periods with the wash buffer, and after the removal of unbound secondary antibody the membrane was further washed in 10 mM PBS for five 10-min periods. The presence of horseradish peroxidase was then demonstrated using an enhanced chemiluminescent technique (Amersham International), which was performed as described previously (37).

Immunohistochemistry. Immunohistochemistry was used to determine the cellular localization and distribution of CYP1B1 and was performed on formalin-fixed, wax-embedded sections. Sites of immunoreactivity were detected using an alkaline phosphatase antialkaline phosphatase technique (40). Sections of tumor or normal tissues were cut and mounted on amino-propyl-ethoxy silane (Sigma)-coated glass slides to ensure section adherence. Sections were dewaxed in xylene, rehydrated in alcohol, and then washed sequentially in cold water and TBS[3] [0.05 M Tris-HCl (pH 7.6) containing 0.15 M sodium chloride]. The sections were then immunostained with the CYP1B1 antibody (1:250 in TBS). Subsequently, monoclonal mouse antirabbit immunoglobulin (1:100; DAKO Ltd., High Wycombe, Bucks, United Kingdom), rabbit anti-mouse immunoglobulin (1:100; DAKO), and mouse monoclonal alkaline phosphatase antialkaline phosphatase (1:100; DAKO) were applied sequentially to the tissue sections for 30 min each. Between antibody applications, the sections were washed with TBS for three successive 5-min periods to remove unbound antibody. Sites of bound alkaline phosphatase were demonstrated colorimetrically using a solution containing 3 mg of 5-bromo-4-chloro-3-indolyl phosphate (Sigma), 10 mg of nitroblue tetrazolium (Sigma), 6 mg of sodium azide, and 4 mg of levamisole (Sigma) to inhibit endogenous alkaline phosphatase in 10 ml of 0.05 M Tris-HCl buffer, pH 9.0, containing 0.2% magnesium chloride. After the sections were incubated for 30 min at room temperature, the enzyme reaction was stopped by washing the sections for 5 min in cold tap water. The slides were then air dried and mounted in glycerine jelly. The sections were examined using bright field light microscopy to establish the presence or absence of immunostaining and its distribution.

[3] The abbreviation used is: TBS, Tris-buffered saline.

RESULTS

CYP1B1 Antibody. The anti-CYP1B1 antibody that was generated using the synthetic peptide corresponding to the CYP1B1 specific sequence (Fig. 1) recognized expressed CYP1B1 (Fig. 2) and a single band of identical molecular size (M_r 52,000) in breast cancer microsomes. The antibody did not recognize expressed CYP1A1 or any protein in human liver microsomes. The liver microsomes have been shown previously to contain CYP1A2 (41).

Immunohistochemistry. Immunohistochemistry for CYP1B1 showed that CYP1B1 immunoreactivity was detected in all of the different types of cancer studied and that there was strong CYP1B1 immunoreactivity in every type of tumor (Table 1; Figs. 3 and 4). In each type of tumor, CYP1B1 was expressed at high frequency, and only in 5 of 127 tumors was CYP1B1 not detected. In every type of cancer, CYP1B1 immunoreactivity was localized specifically to tumor cells (Figs. 3 and 4); nontumor cells, including inflammatory cells and endothelial cells present in the sections of tumor, showed no apparent immunoreactivity for CYP1B1. There was no significant intratumor heterogeneity of CYP1B1 immunoreactivity and no detectable immunoreactivity for CYP1B1 in any of the normal tissues studied, including liver, kidney, and small intestine (Table 1; Fig. 3).

DISCUSSION

The absence or low levels of individual forms of cytochrome P450 in many studies of human cancer (25–32), combined with extrapola-

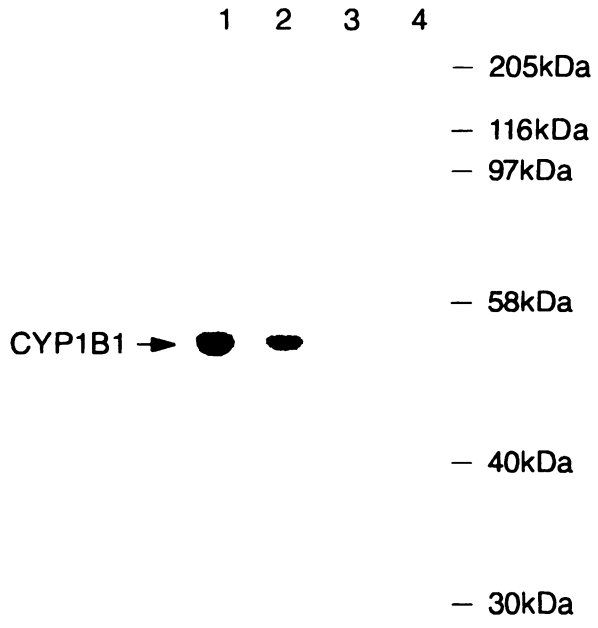

Fig. 2. Immunoblot of microsomes from human lymphoblastoid cells expressing CYP1B1 (*Lane 1*), human breast cancer microsomes (*Lane 2*), microsomes from human lymphoblastoid cells expressing CYP1A1 (*Lane 3*), and normal human liver microsomes (*Lane 4*). The anti-CYP1B1 antibody recognizes recombinant CYP1B1 and CYP1B1 present in breast cancer microsomes and does not recognize either expressed CYP1A1 or any protein in liver microsomes. Ten μg of protein were loaded per lane; in *Lane 1*, this corresponds to 0.74 pmol of expressed CYP1B1. *Right*, position of molecular weight standards.

Table 1 *Expression of CYP1B1 in different types of malignant tumors and normal tissue*

Tissue	Normal (No. positive/no. tested)	Tumor [no. positive/no. tested (histopathological diagnosis)]
Bladder	0/8	8/8 (transitional cell carcinoma)
Brain	0/12	11/12 (astrocytoma)
Breast	0/10	12/12 (invasive ductal carcinoma)
Colon	0/10	11/12 (adenocarcinoma)
Connective tissue	0/9	8/9 (sarcoma)
Esophagus	0/8	8/8 (squamous carcinoma)
Kidney	0/11	11/11 (clear cell carcinoma, n = 10; transitional cell carcinoma, n = 1)
Liver	0/8	Not tested
Lung	0/8	7/8 (squamous carcinoma)
Lymph node	0/5	9/9 (non-Hodgkin's lymphoma)
Ovary	0/5	7/7 (adenocarcinoma)
Skin	0/6	6/6 (squamous carcinoma)
Small intestine	0/5	Not tested
Stomach	0/10	9/10 (adenocarcinoma)
Testis	0/8	8/8 (malignant germ cell tumor)
Uterus	0/7	7/7 (adenocarcinoma, n = 5; malignant mixed Müllerian tumor, n = 2)
Total	0/130	122/127

tion from studies of rodent hepatic carcinogenesis (42–44), has led to the general belief that tumor cells do not significantly express cytochrome P450. However, this study has shown that CYP1B1 is expressed in a wide variety of malignant tumors of different histogenetic types but is not detectable in normal tissues, indicating that this cytochrome P450 is a tumor-specific form of cytochrome P450. The presence and cellular localization of CYP1B1 was investigated through immunohistochemistry with an antibody specific for CYP1B1 in different types of cancers that had developed in a variety of anatomical sites. Primary malignant tumors of these tissues constitute different histogenetic types (carcinomas, lymphomas, sarcomas, neuroepithelial tumors, and germ cell tumors), each with a different

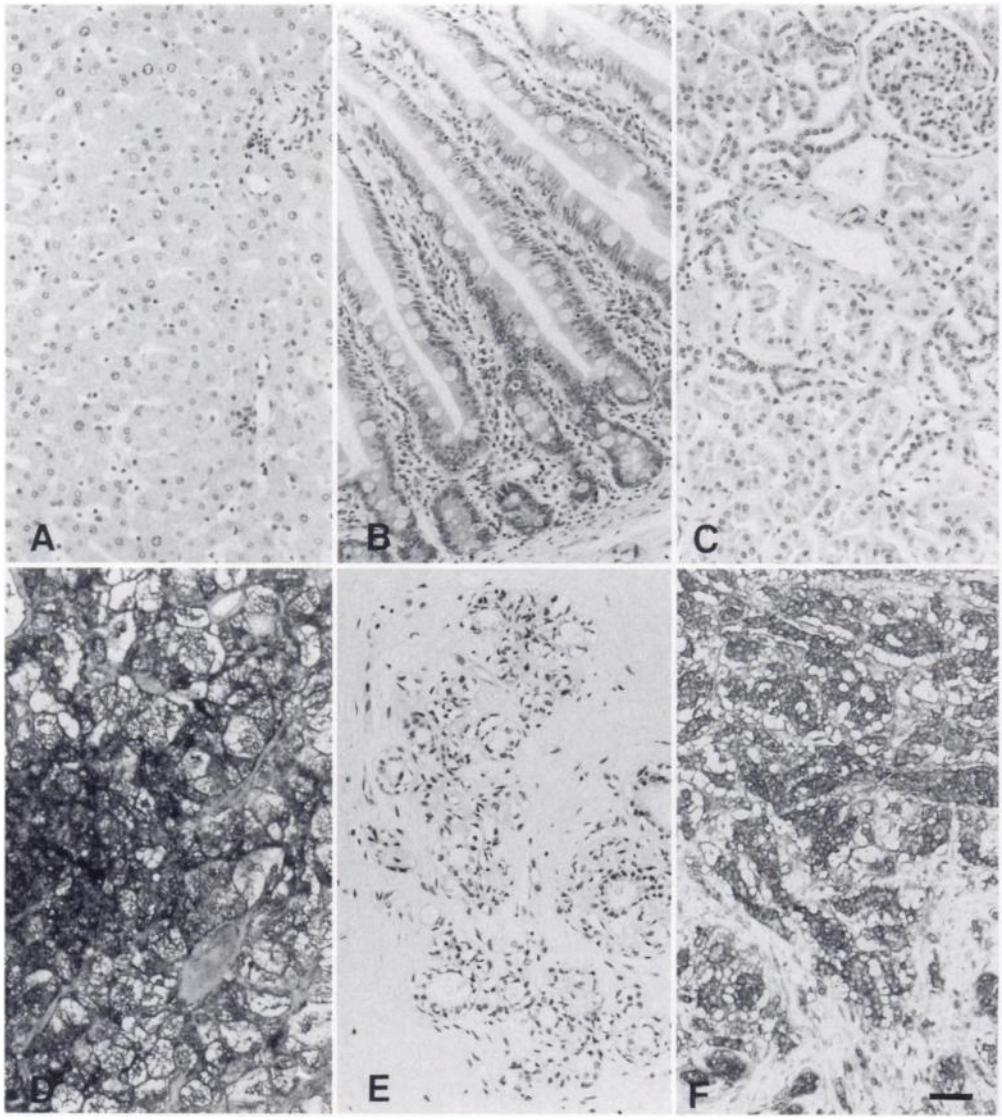

Fig. 3. Immunohistochemical localization of CYP1B1 in tumors and normal tissues. In tumors, CYP1B1 is specifically localized to tumor cells, and there is no CYP1B1 immunoreactivity in normal tissues. *A*, normal liver; *B*, normal small intestine, *C*, normal kidney; *D*, carcinoma of kidney; *E*, normal breast; *F*, invasive ductal carcinoma of breast (the normal tissues in panels *A*, *B*, *C*, and *E* have been stained with hematoxylin to show histological detail). ×300. *Scale bar*, 40 μm.

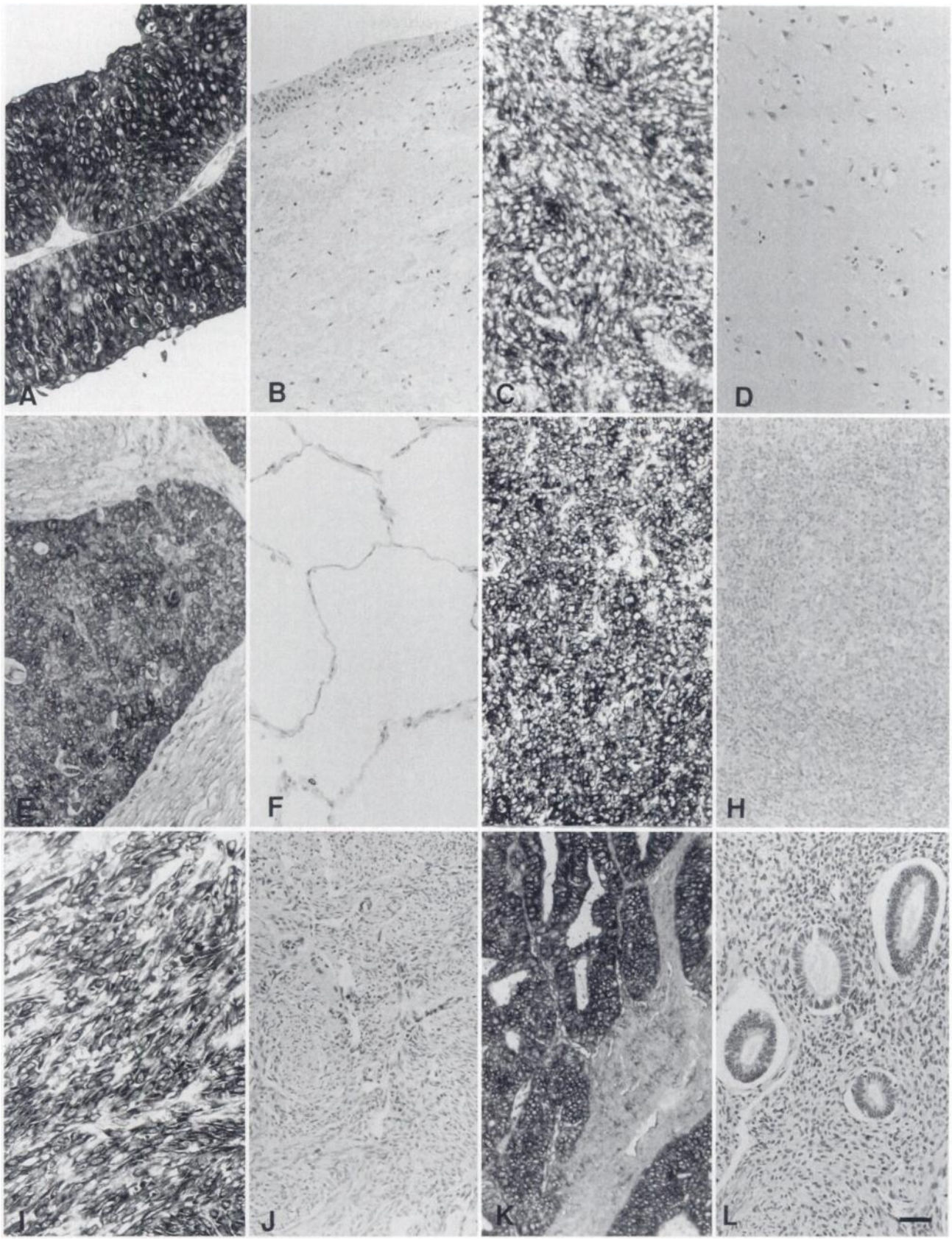

Fig. 4. Immunohistochemical localization of CYP1B1 in different types of malignant tumors and normal tissues. CYP1B1 immunoreactivity is present in tumors and absent from normal tissues. *A*, transitional cell carcinoma of bladder; *B*, normal bladder; *C*, high grade astrocytoma of brain; *D*, normal brain; *E*, non-small cell carcinoma of lung; *F*, normal lung; *G*, malignant lymphoma; *H*, normal lymph node; *I*, leiomyosarcoma of uterus; *J*, normal uterus-myometrium; *K*, endometrial adenocarcinoma; *L*, normal uterus-endometrium. ×300. *Scale bar*, 40 μm.

biological behavior. These tumors also represent a range of both common and less common types of cancer. Immunohistochemistry ensures the identification of specific types of cells containing CYP1B1 and is an ideal technique for investigating the presence of CYP1B1 in tumor cells, because tumors are composed of a variable proportion of tumor cells and nontumor cells. Moreover, many tumors may only be available as formalin-fixed, wax-embedded sections.

The presence of CYP1B1 in many types of cancer suggests that this cytochrome P450 may have a crucial endogenous function in tumor cells and that CYP1B1 might also contribute to drug resistance that is observed in many types of cancer. CYP1B1 may also be important in tumor development and progression, because human CYP1B1 expressed in yeast has been shown to be capable of metabolizing a variety of putative human carcinogens (45). In addition, CYP1B1 has been shown to be capable of 4-hydroxylation of estradiol (46–48), which may be important in the development of cancers in estradiol-dependent tissues. Although CYP1B1 mRNA has been identified in a limited number of normal tissues (16, 45), our findings of an absence of detectable CYP1B1 protein in normal tissues suggest that either CYP1B1 protein is present at a very low level in normal tissues or the CYP1B1 mRNA is not translated. Recently, significantly increased 4-hydroxylation of estradiol has been identified in breast cancer (49). The expression of CYP1B1 in tumors is likely to involve several regulatory mechanisms. Regulation of the expression of forms of cytochrome P450 in liver is complex; multiple mechanisms, including transcriptional and posttranscriptional factors, are involved.

The expression of CYP1B1 in different types of malignant tumors has important consequences for both the diagnosis and treatment of cancer. New diagnostic procedures based on the presence of CYP1B1 in cancer cells can be developed, whereas the expression of CYP1B1 in tumor cells provides a molecular target for the development of new anticancer drugs that could be selectively activated by the presence of CYP1B1 in tumor cells.

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