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Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood–brain barrier sites

(monoclonal antibodies/immunohistochemistry/cancer chemotherapy)

CARLOS CORDON-CARDO*, JAMES P. O'BRIEN*, DOLORS CASALS*, LANA RITTMAN-GRAUER†, JUNE L. BIEDLER*, MYRON R. MELAMED*, AND JOSEPH R. BERTINO*

*Memorial Sloan–Kettering Cancer Center, 1275 York Avenue, New York, NY 10021; and †Hybritech, Inc., 11095 Torreyana Road, San Diego, CA 92121

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ABSTRACT **Endothelial cells of human capillary blood vessels at the blood–brain and other blood–tissue barrier sites express P-glycoprotein as detected by mouse monoclonal antibodies against the human multidrug-resistance gene product. This pattern of endothelial cell expression may indicate a physiological role for P-glycoprotein in regulating the entry of certain molecules into the central nervous system and other anatomic compartments, such as the testes. These tissues, which limit the access of systemic drugs, are known pharmacologic sanctuaries for metastatic cancer. P-glycoprotein expression in capillary endothelium of brain and testes and not other tissues (i.e., kidney and placenta) may in part explain this phenomenon and could have important implications in cancer chemotherapy.**

The failure of chemotherapy to eradicate all cancer cells from a patient may be due to a variety of causes. The ability of some cancer cells to develop drug resistance is one such possibility (1). Recently, significant progress has been made in understanding several mechanisms of drug resistance by using cultured tumor cells selected for resistance to a given drug. Multidrug resistance (mdr) is the term used to describe the broad pattern of cross-resistance that is seen following the development of resistance to certain cytotoxic natural products (2, 3). Multidrug-resistant cells consistently overexpress a unique membrane glycoprotein (M_r, 170–180), P-glycoprotein (Pgp), which is encoded by the mdr1 gene (4). Based on the homology of the mdr1 gene to a variety of genes encoding membrane transport proteins (4) and on the ability of Pgp to bind drugs (5) and hydrolyze ATP (6), it is currently believed that mdr results from the function of Pgp as an energy-dependent drug efflux pump.

Expression of Pgp has been reported in a variety of human tumors (7) and in normal human tissues (8, 9). Two main types of normal cells highly express this transmembrane product: (*i*) specialized epithelial cells with secretory/excretory functions (e.g., epithelial cells lining the gastrointestinal tract and kidney) (8) and (*ii*) trophoblasts in the placenta (9). We now report the expression of Pgp in endothelial cells of capillary blood vessels at blood–brain and other blood–tissue barrier sites.

MATERIALS AND METHODS

Immunocytochemical Technique. The human melanoma cell line BRO was transfected with a human mdr1 cDNA under control of a cytomegalovirus promoter and hepatitis B virus polyadenylation signals (parental and mdr1-transfected BRO cell lines were supplied by Piet Borst, The Netherlands Cancer Institute). Mouse monoclonal antibodies (mAbs) HYB-241 and HYB-612 were supplied by Hybritech (San Diego, CA). These antibodies are mouse immunoglobulins of the IgG1 class. They were utilized as purified immunoglobulin preparations at ≈20 μg/ml in phosphate-buffered saline (PBS) containing 2% bovine serum albumin (PBS/BSA). Mouse mAb C219 was used for the present study as a purified immunoglobulin preparation at 20 μg/ml (Centocor, Malvern, PA). As a negative control, purified mouse mAb directed against the anthranilate synthase of the *Escherichia coli* was used at the same working dilutions (bcr-25, Oncogene Science, New York; 20 μg/ml). As a positive control, purified mAbs against cytokeratins and other intermediate filaments (e.g., simentin) were also used at the same concentrations (Cambridge Research Laboratories; 20 μg/ml). Secondary antibodies used were (*i*) goat anti-mouse IgG (γ- + light-chain specific) affinity-purified antibodies, peroxidase-conjugated (1:50 dilution in PBS/BSA) (Tago); and (*ii*) biotinylated horse anti-mouse IgG (heavy- + light-chain specific) affinity-purified antibodies (1:100 dilution in PBS/BSA) followed by avidin–biotin–peroxidase complexes (1:100 dilution in PBS) (Vector Laboratories). Immunocytochemistry was performed with fresh cells grown on microslides and fixed with cold acetone for 10 min. Both indirect immunoperoxidase and avidin–biotin complex methods were used as described (10, 11). The secondary antibodies were used as indicated above. Diaminobenzidine was used as chromogen (5 mg of diaminobenzidine tetrahydrochloride in 100 ml of PBS with 100 μl of 0.3% hydrogen peroxide). The diaminobenzidine solution was filtered and incubated with the tissue sections for 6–12 min. After treatment, cells were washed with distilled H_2O, counterstained with hematoxylin, and mounted with permount.

Immunohistochemical Technique. Histologically normal adult human tissues were obtained from surgical pathology specimens within 1–2 hr of resection, and autopsy specimens were obtained within 10 hr of death. Several normal specimens from each organ site and different cases, ranging from 2 to 12 samples, were used for the present study. Tissues were histologically well preserved. Fresh tissues were immersed in isopentane precooled in liquid nitrogen, embedded in OCT compound in cryomolds (Miles), and stored at −70°C until needed. Frozen sections were fixed for 10 min in cold acetone and then quenched in 0.1% hydrogen peroxide in PBS for 10 min. Tissue sections then were washed several times in PBS and incubated with normal horse blocking serum at 10% dilution in PBS/BSA for 20 min. The blocking serum was removed and the sections were incubated with appropriately diluted primary antibody. Immunohistochemical stainings were performed as described above and elsewhere (10, 11).

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Abbreviations: mdr, multidrug resistance; Pgp, P-glycoprotein; mAb, monoclonal antibody; BSA, bovine serum albumin.

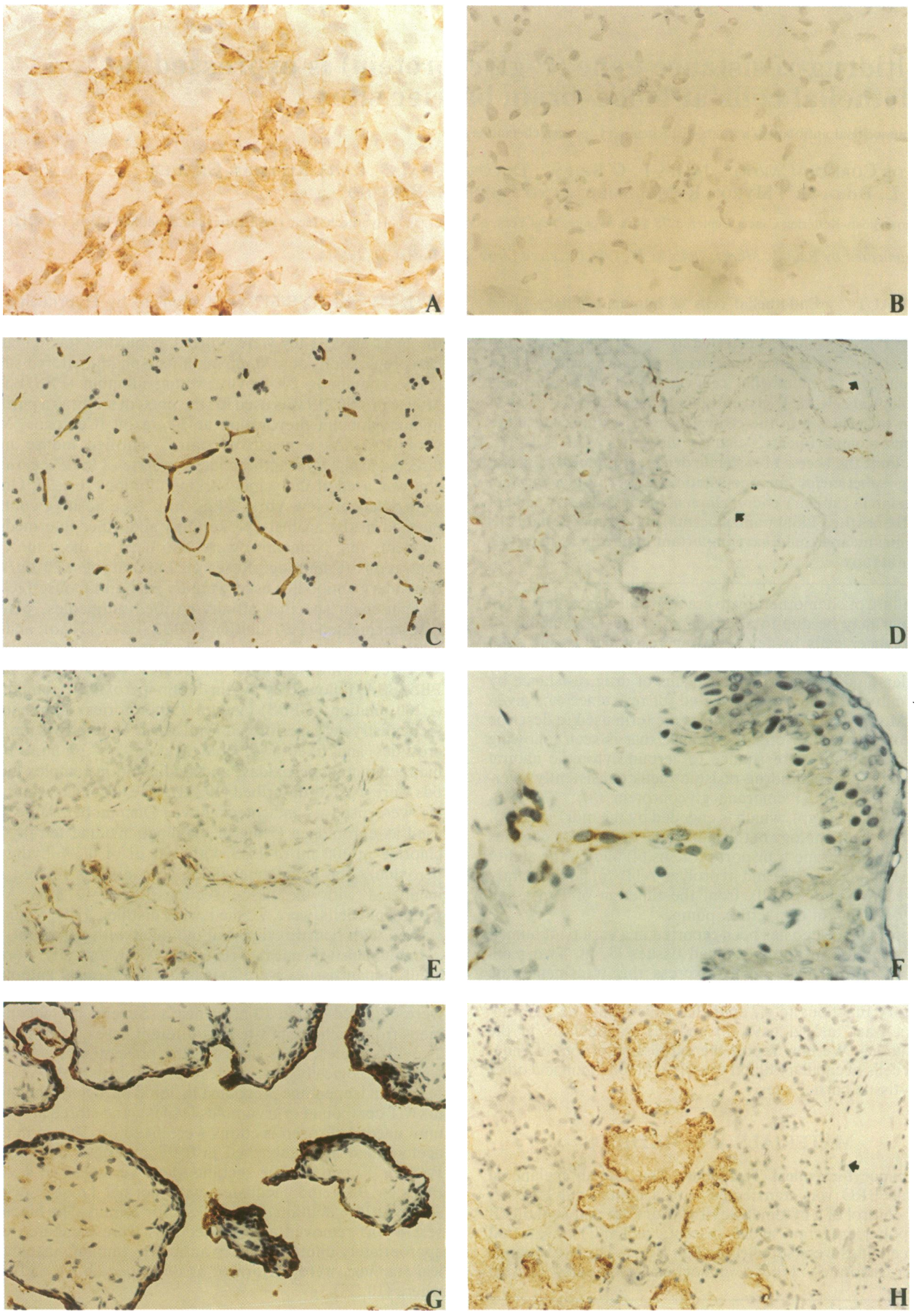

FIG. 1. (*Legend appears at the bottom of the opposite page.*)

RESULTS AND DISCUSSION

As a means of further characterizing the normal tissue distribution of Pgp, three mouse mAbs directed against at least two different epitopes of the human mdr1 gene product were used in immunocytochemical and immunohistochemical studies. The mAbs HYB-241 and HYB-612 (12, 13) bind to an extracellular Pgp epitope, whereas C219 (14) recognizes an internal portion of the molecule. To corroborate the specificity of HYB-241, this antibody was tested for its immunoreactivity on cells made multidrug resistant by transfection with the human mdr1 gene, as well as on the drug-sensitive parental cell line. The drug-resistant mdr1-transfected cells were strongly immunoreactive, while the parental cell line was not (Fig. 1 *A* and *B*).

Normal human tissues were analyzed for the expression of Pgp. Cells containing detectable levels of this product were found in most of the secretory organs surveyed, as reported (8). In addition, we observed that other specialized epithelial cells such as the eccrine or sweat glands in the skin, cells lining the trachea and major bronchi in the lung, acinar cells of the pancreas, follicles of the thyroid, and scattered foci of cells of the mammary gland, endometrium, and prostate have detectable expression (data not shown). The most intriguing observation, however, is the strong expression of Pgp found on endothelial cells of capillary blood vessels at blood–tissue barrier sites. These anatomical locations are (*i*) capillaries of the central nervous system (including cerebral cortex, cerebellum, and spinal cord) (Fig. 1 *C* and *D*), (*ii*) capillaries of the testes (Fig. 1*E*), and (*iii*) capillaries of the papillary dermis (Fig. 1*F*) (see Table 1).

Capillary endothelium of the choroid plexus is nonreactive and there is no staining in capillaries of other organs (i.e., placenta and kidney) (Fig. 1 *G* and *H*) (Table 1). The endothelium of arterioles and larger blood vessels is unreactive for the three mAbs. Homogeneous strong immunoreactivity was observed in placental trophoblast, while capillary endothelial cells at this site were unreactive (Fig. 1*G*). No qualitative or quantitative differences in the pattern of staining were found among the three antibodies used in the tissues reported here.

The identification of Pgp expression by capillary endothelial cells at blood–brain and other blood–tissue barrier sites raises the possibility that this protein may be an operative component of these systems. The blood–brain barrier is anatomically characterized by the presence of intercellular tight junctions between continuous nonfenestrated endothelial cells, which normally function to limit the passage of protein and soluble polar compounds into the brain parenchyma (15). While certain nonpolar molecules such as ethanol, nicotine, and heroin readily enter the central nervous system, it is not well understood how the brain is protected from the passive inward diffusion of other harmful nonpolar molecules, such as certain xenobiotics, which may enter the circulation. The restricted entry of these molecules is demonstrated by the 15 to 30 times lower cerebrospinal fluid drug levels for actinomycin D (16) and vincristine (17) as compared to simultaneous plasma levels. The concept of the blood–brain barrier is not limited to specific microanatomical features but is expanding to accommodate the many newly recognized functions of this unique capillary system. In addition to the previously described specialized functions of the central nervous system capillary endothelium (18–20), these cells are now further distinguished by the presence of a cell-surface protein, which in tissue culture models has been shown to bind and export cytotoxic substances such as doxorubicin, actinomycin D, and vincristine.

The relapse of acute lymphocytic leukemia in the testes (21) or the meninges (22) following remission achieved by systemic chemotherapy is a well-documented occurrence. This is believed to be the result of malignant cells surviving induction chemotherapy because of the failure of these drugs to penetrate into the testes (23) or the cerebrospinal fluid (24). The expression of Pgp in the capillary network of the testes and brain is consistent with this clinical observation.

Table 1. Distribution of Pgp in human capillary endothelial cells detected by HYB-241 and C219 mouse mAbs

Tissue type	HYB-241	C219
Nervous system		
Frontal cortex	+	+
Hippocampus	+	+
Cerebellum	+	+
Spinal cord	+	+
Choroid plexus	−	−
Meninges	−	−
Peripheral nerves	−	−
Sympathetic ganglia	−	−
Reproductive system		
Testes	+	+
Rete testis	−	−
Ovaries	−	−
Uterus	±	±
Skin		
Papillary dermis	+	+
Reticular dermis	−	−
Hematopoietic system		
Lymph node	−	−
Spleen	−	−
Thymus	−	−
Gastrointestinal system		
Stomach	−	−
Colon	−	−
Repiratory system		
Bronchi	−	−
Lung	−	−
Urinary system		
Kidney	−	−
Ureter	−	−
Urinary bladder	−	−
Placenta	−	−

Analysis of fresh frozen tissue sections by immunoperoxidase techniques. Endothelial cells of arterioles and larger blood vessels were unreactive in these microanatomical locations. +, Homogeneously stained; ±, heterogeneously stained; −, undetectable immunoreactivity.

FIG. 1 (*on opposite page*). (*A*) BRO melanoma cells transfected with human mdr I gene product and stained with mAb HYB-241 by the avidin–biotin–peroxidase technique. (×100.) Note the strong immunoreactivity pattern observed in these transfected cells. (*B*) BRO nontransfected melanoma cells stained as in *A* with mAb HYB-241. (×100.) (*C*) Human frontal cortical brain tissue showing immunoperoxidase-positive staining of capillary endothelial cells with mAb HYB-241. (×100.) Results with HYB-612 and C219 were identical in pattern and intensity of immunohistochemical staining (data not shown). (*D*) Endothelial cells of meningeal blood vessels were unreactive for HYB-241 (arrow). However, capillaries in the brain were strongly positive. (×20.) (*E*) Capillary endothelium of testes showing immunoreactivity. Germinal cells in the adjacent seminiferous tubules were unreactive. (×100). (*F*) Endothelial cells of capillaries of the papillary plexus were intensively positive for HYB-241. Epidermis and other dermal mesenchymal cells were unreactive. (×200.) (*G*) Human placental trophoblastic cells showing immunoperoxidase-positive staining with mAb HYB-241. Capillary endothelial cells in this tissue were unreactive. (×100.) Again, results of immunohistochemical staining with HYB-612 and C219 were identical (data not shown). (*H*) Epithelial cells of the proximal tubules of the kidney were immunoreactive. Note the lack of reactivity with capillaries of the glomerular tuft and endothelial cells of the arteriole (arrow). (×100.)

The skin is generally not thought of as a blood–tissue barrier site; however, several interesting aspects of the dermal microcirculation deserve consideration in this regard. The capillaries of the dermis are divided into three separate plexuses, one each surrounding hair follicles and eccrine glands and one localized to the papillary dermis (25). It is in the capillaries of the papillary dermis where Pgp expression is detected and not on the endothelial cells of the eccrine gland and hair follicle plexuses. While no data exist regarding the penetration of chemotherapeutic agents into the microanatomic compartments served by each of these capillary beds, it is interesting to note that the cutaneous toxicities of natural product cytotoxic agents are generally limited to the skin appendages (e.g., alopecia), whereas there is no apparent suppression of the rapidly proliferating epidermal keratinocytes. It is on the endothelium of the papillary plexus, which subtends the epidermis, that Pgp expression is detected and not on other dermal capillary endothelial cells. This clinical–pathological correlation, along with the finding of a continuous nonfenestrated endothelium in the papillary dermal capillary loops (26), provides a link between the observation of Pgp expression in these capillaries and those of the brain and testis.

We therefore propose that this pattern of endothelial cell expression may indicate a physiologic role for Pgp in regulating the entry of certain molecules into specific anatomic compartments, a function that may in part explain the failure of systemic chemotherapy to eradicate otherwise drug-sensitive tumor cells that may be present in such known pharmacologic sanctuaries as brain and testis.

When developing therapeutic strategies for suppression of the Pgp mechanism in drug-resistant tumor cell populations, it will be important to anticipate possible host toxicities that may result from interference with the protective function of blood–organ barriers. Facilitating the entry of potent neurotoxins, such as vinca alkaloids, into the central nervous system would be a highly undesirable side effect of any treatment regimen. Alternatively, careful and creative approaches to therapeutic design may allow the manipulation of blood–organ barriers so as to abolish pharmacologic sanctuaries and increase the efficacy of chemotherapy without excess host toxicity.

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