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Tissue Distribution of P-Glycoprotein Encoded by a Multidrug-resistant Gene as Revealed by a Monoclonal Antibody, MRK 16

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ABSTRACT

A monoclonal antibody, MRK 16, specific to a human myelogenous leukemia cell line, K-562, and resistant to Adriamycin, was used to determine the localization of the antigen molecules (P-glycoprotein) recognized by the monoclonal antibody. P-glycoprotein was found to be expressed very strongly in the adrenal cortex and medulla of adults and strongly in the renal tubules of the kidney and the placenta. Interestingly, P-glycoprotein was not distributed in fetal and neonatal adrenals, and thus may be closely related to adrenal maturation. A high level of P-glycoprotein expression was also seen in one case each of untreated lung cancer (one of ten) and breast cancer (one of nine).

Immunoelectron microscopically, the P-glycoprotein was distributed evenly on the membranes of K-562/ADM and 2780 cells.

These results imply that the presence of the glycoprotein may be useful as a marker for *in vitro* studies of multidrug resistance in various malignancies and as an indicator of therapeutic efficacy of *ex vivo* eradication of multidrug-resistant cancer cells, although other mechanisms of drug resistance may exist, and there is a possibility that this MRK 16 monoclonal antibody may not recognize all P-glycoprotein.

INTRODUCTION

Adriamycin has been widely used for the treatment of solid tumors such as lung cancer, breast cancer, and ovarian cancer, as well as acute leukemia and malignant lymphoma (1). Therefore, it is of vital importance to predict the development of drug resistance as early as possible.

It has recently been reported that Adriamycin-resistant leukemia, Adriamycin-resistant and daunomycin-resistant Chinese hamster ovary cell mutants, and *Vinca* alkaloid-resistant human leukemic lymphoblasts exhibit a M_r 170,000 to 180,000 glycoprotein (P-glycoprotein) which is hardly recognized in their parental cells (2–6). The glycoprotein, 1280 amino acids long in humans and 1276 amino acids long in mice, consists of two homologous parts of approximately equal length (7, 8).

In order to understand the mechanism of multidrug resistance, we obtained an Adriamycin-resistant leukemia cell line (K-562/ADM) and subsequently prepared murine MAbs[2] specific to K-562/ADM, MAbs MRK 16 and 17 (6, 9). Both MAbs specifically recognized the M_r 170,000 to 180,000 glycoprotein (P-glycoprotein) in various human multidrug-resistant tumor cell lines (10).

As a further step, the present experiments were designed to evaluate the level of P-glycoprotein expression by one of the MAbs, MRK 16, utilizing both normal and cancerous tissues.

It was found that the P-glycoprotein was stained very strongly with MRK 16 in adult adrenals and strongly in kidney and placenta, and in one case each of lung carcinoma and breast carcinoma, as assessed by the indirect immunoperoxidase technique.

MATERIALS AND METHODS

Cell Lines and Tissues. The cell lines K-562 (human myelogenous leukemia) and Adriamycin-resistant K-562 (K-562/ADM) were established in the laboratory of one of the authors (6). An ovarian cancer cell line, A2780, and its Adriamycin-resistant strain, 2780^{AD}, were kindly supplied by Dr. R. F. Ozols and Dr. T. C. Hamilton, National Cancer Institute (11, 12). A rat pheochromocytoma cell line (P-12) was kindly provided by Dr. M. Noda, Riken Institute, Japan (13). All of the cell lines were grown in RPMI 1640 medium containing 10% fetal calf serum and gentamicin sulfate (20 μg/ml). The cell lines were routinely passaged twice weekly.

For immunohistochemical study, human tissues were obtained from biopsies performed on patients for diagnostic procedures at Saitama Medical University Medical Center and the University of Tokyo Hospital, and from autopsies performed within 2 h of death on patients with various heart diseases and malignancies. Tissues for frozen section studies were immediately snap frozen in liquid nitrogen and stored at −70°C until sectioning.

Preparation and Characterization of Monoclonal Antibody MRK 16. The methods used for antigen preparation, immunization, cell fusion, cloning, and serological characterization of monoclonal antibody (MRK 16) isotype have been previously described in detail (9).

Immunoperoxidase Staining Method. Frozen sections were prepared for immunoperoxidase staining as described previously (14). For these experiments, 100 μl of MRK 16 MAb (5 μg/ml) and nonimmune mouse serum (100 μg/ml) as a negative control were used.

Ultrastructural Localization of P-Glycoprotein in K-562/ADM and 2780^{AD} Cells. To examine the localization of the P-glycoprotein recognized by MRK 16 MAb, an immunoelectron microscopical study was performed. K-562, K-562/ADM, A2780, and 2780^{AD} cells (1×10^7/ml) in suspension were first treated with PLP for 45 min and then with 0.5% saponin (Sigma Chemical Co., St. Louis, MO) in PBS for 2 h. Thereafter, they were treated with MRK 16 MAb (100 μg/ml) at 4°C for 12 h. After being washed carefully with PBS 3 times, 50 μl of peroxidase-labeled rabbit anti-mouse IgGs (DAKO PATTS, Copenhagen, Denmark) were applied. The cells were fixed with 2.5% glutaraldehyde-PBS for 20 min after frequent washing with PBS. Thereafter, 500 μl of 3,3-diaminobenzidine-H_2O_2 solution (2.5 mg of 3,3-diaminobenzidine and 10 μl of H_2O_2 in PBS) were added for 10 min for coloration. After three washings with PBS, the cells were postfixed with 1% osmic acid solution for 30 min and embedded in Epon 812. Ultrathin sections were prepared using an ultramicrotome (ULTRACUT E; REICHERT-JUNG Co.). These were then stained with uranyl acetate and examined with an electron microscope (Model 100C; JEOL, Tokyo, Japan) (15).

RESULTS

Immunoperoxidase Staining Reactivity of MRK 16 MAb with Human Normal Tissues. In order to assess the binding reactivity of MRK 16 MAb with normal human tissues, frozen sections of lymphoid and nonlymphoid organs were stained with MRK 16 MAb using the immunoperoxidase technique (indirect

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[2] The abbreviations used are: MAb, monoclonal antibody; PBS, phosphate-buffered saline; ADM or AD, Adriamycin; EGF, epidermal growth factor; PLP, periodate-lysine-paraformaldehyde fixative.

method). This reacted only with the cell membrane of K-562/ADM (Fig. 1) and 2780^{AD}, but not with K-562 and A2780 (Table 1). Interestingly, it also reacted with some cells in the adrenal zona glomerulosa, zona fasciculata, and zona reticularis and some cells in the medulla which had a tendency to appear in cell clusters (Table 1; Fig. 2), some placental trophoblasts (Fig. 3), and some kidney proximal tubules.

Since adult adrenals were stained very positively, we also used samples of fetal adrenal (20 wk of gestation) and neonatal adrenal (3 days after birth) for comparison. Contrary to our expectation, the MAb did not stain the fetal and neonatal adrenals. Furthermore, it did not react with cell line PC-12.

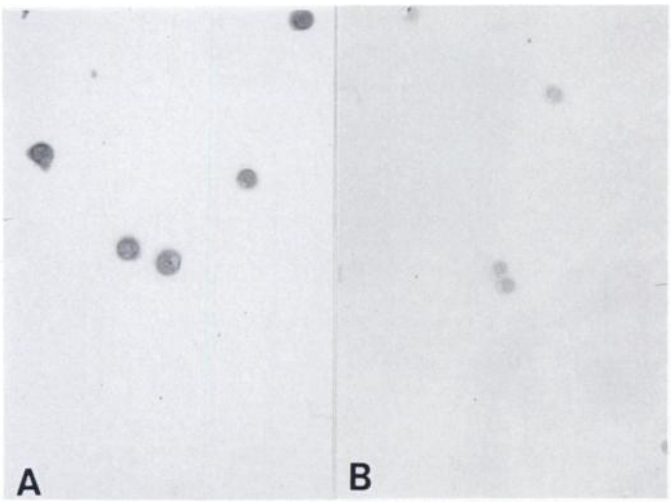

Fig. 1. Immunostaining of P-glycoprotein on K-562/ADM (*A*) and K-562 (*B*) cells by MRK 16 MAb. × 800.

Table 1 *Reactivity of MRK 16 MAb with normal tissue cell lines*

Tissue or cell line	Reactivity of MRK 16 MAb
Adult adrenal	++[a](5/5)[b]
Fetal adrenal	− (0/1)
Neonatal adrenal	−(0/1)
Full-term placenta	+ (2/2)
Kidney	+ (5/5)
Pancreas	− (0/3)
Rectum	− (0/2)
Gallbladder	− (0/1)
Thyroid	− (0/1)
Lymph node	− (0/2)
Liver	− (0/2)
Prostate	− (0/2)
Stomach	− (0/2)
Lung	− (0/3)
Submandibular gland	− (0/2)
Pituitary	− (0/2)
Mammary gland	− (0/2)
Large intestine	− (0/2)
Small intestine	− (0/2)
Spleen	− (0/1)
Heart (left ventricle)	− (0/1)
Skeletal muscle (femoral part)	− (0/1)
Cerebrum	− (0/2)
Cerebellum	− (0/2)
Esophagus	− (0/2)
Forearm skin	− (0/2)
Femoral bone marrow	− (0/1)
PC-12 (rat pheochromocytoma)	−
K-562 (human myelogenous leukemia)	−
K-562/ADM (Adriamycin-resistant K-562 cells)	++
A2780 (human ovarian carcinoma)	−
2780^{AD} (Adriamycin-resistant A2780 cells)	+

[a] Intensity if indirect immunoperoxidase staining is classified as follows: −, negative; +, positive if less than 50% of the tissues or cells is stained; ++, strongly positive if most of the tissues or cells is stained.
[b] Numbers in parentheses, number of cases.

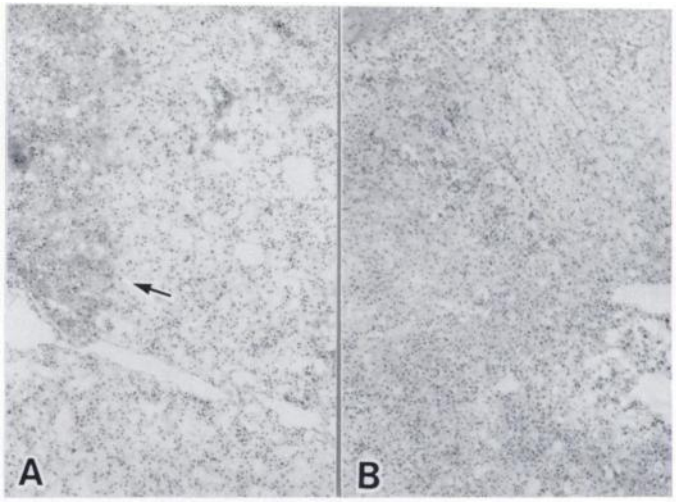

Fig. 2. Immunostaining of P-glycoprotein on adult adrenal. Note that both cortex (*arrow*) and medulla are stained with MRK 16 positively and unevenly (*A*), while no positive staining with appropriately diluted nonimmune mouse serum instead of MRK 16 is noted (*B*). × 300.

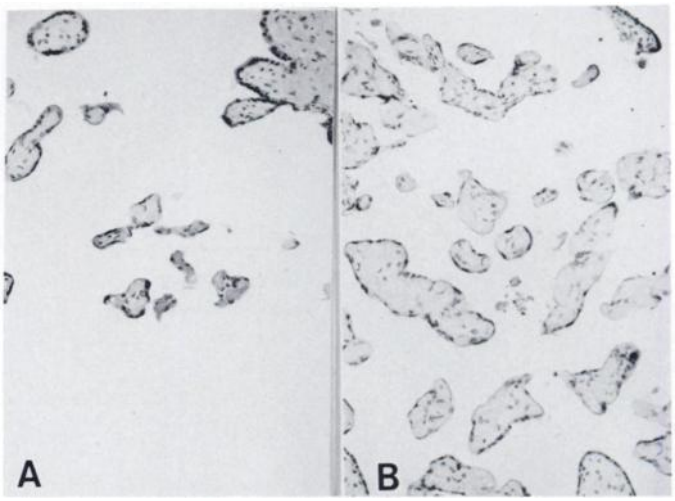

Fig. 3. Immunostaining of P-glycoprotein on placenta. The placenta is stained positive with MRK 16 (*A*), but not with nonimmune mouse serum (*B*).

Staining Reactivity of MRK 16 MAb with Human Malignant Surgical Specimens. No anticancer agents had been administered in these cases. As shown in Table 2 and Figs. 4 and 5, MRK 16 reacted with one of ten lung cancer specimens and one of nine breast cancer specimens. It was noteworthy that not all of the lung and breast cancer cells were stained positively. The histological types showing staining were squamous cell carcinoma and medullary tubular carcinoma, respectively. Also, cancerous tissues expressing P-glycoprotein did not possess EGF receptors.[3] The MAb did not react with frozen sections of eight malignant lymphomas obtained at biopsy.

Ultrastructural Localization of P-Glycoprotein Recognized by MRK 16 in K-562/ADM and 2780^{AD} Cells. As shown in Figs. 6 and 7, P-glycoprotein was distributed abundantly and evenly on the surface membranes of K-562/ADM and 2780^{AD}, but not on K-562 and A2780. Even after treatment with PLP and saponin, the antigen recognized by MRK 16 was localized on the cell membranes of Adriamycin-resistant tumor cells.

[3] Unpublished observation.

Table 2 *Reactivity of MRK 16 MAb with human surgical materials*

Surgical materials	Reactivity of MRK 16
Colon cancer	−[a](0/4)[b]
Pancreas cancer	− (0/2)
Renal cancer	− (0/2)
Malignant lymphoma	− (0/8)
Gastric cancer	− (0/2)
Neuroblastoma	− (0/1)
Malignant fibrous histiocytoma	− (0/1)
Osteogenic sarcoma	− (0/1)
Lung cancer	+ (1/10) (squamous cell carcinoma)
Ovarian cancer	− (0/1)
Uterine cervical cancer	− (0/1)
Smooth muscle sarcoma of the uterus	− (0/1)
Breast cancer	− (1/9) (medullary tubular carcinoma)
Thymoma	− (0/2)
Retinoblastoma	− (0/2)
Hepatoma	− (0/2)
Carcinoid of the bronchus	− (0/1)
Myeloma	− (0/2)

[a] Intensity of indirect immunoperoxidase staining is classified as follows: −, negative; +, positive if less than 50% of the tissues is stained.
[b] Numbers in parentheses, number of case(s).

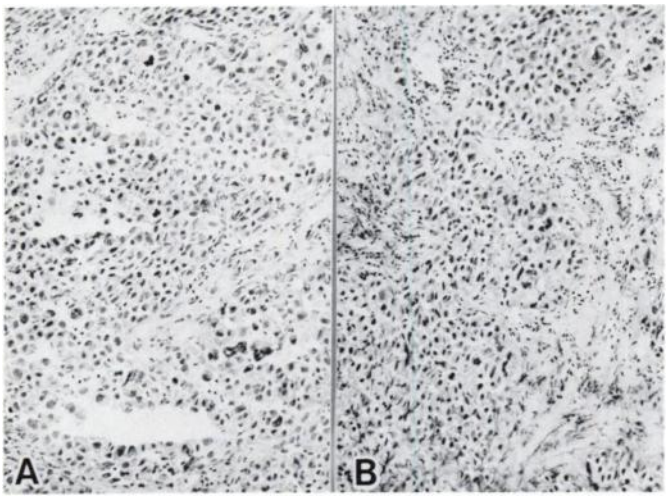

Fig. 4. Immunohistochemical demonstration of P-glycoprotein on untreated lung cancer cells by MRK 16 MAb (*A*). Negative control (nonimmune mouse serum) was used instead of MRK 16 (*B*). × 300.

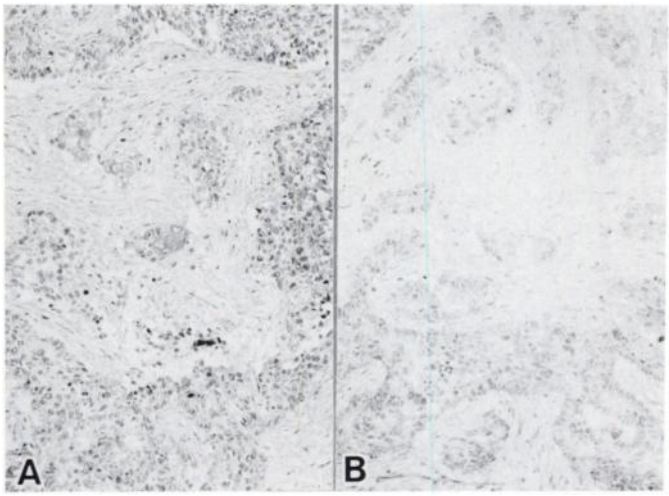

Fig. 5. Immunohistochemical demonstration of P-glycoprotein on untreated breast cancer cells by MRK 16 MAb (*A*). Negative control (nonimmune mouse serum) was used instead of MRK 16 (*B*). × 800.

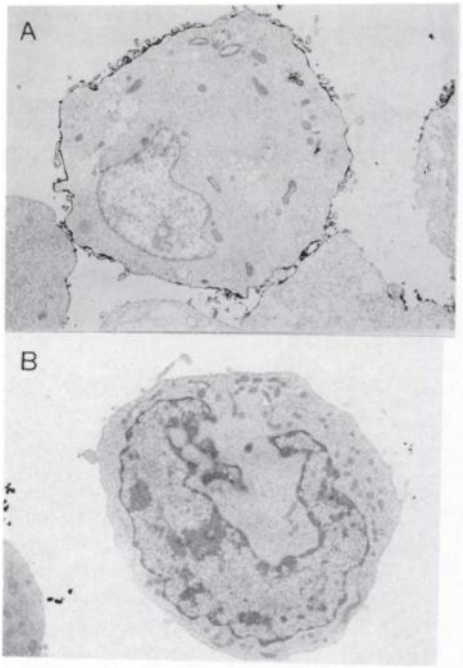

Fig. 6. Ultrastructural localization of P-glycoprotein on K-562/ADM (*A*) and K-562 (*B*) cells by MRK 16 MAb. Note that P-glycoprotein is present on the membranes of K-562/ADM cells, but not on K-562 cells. × 5000.

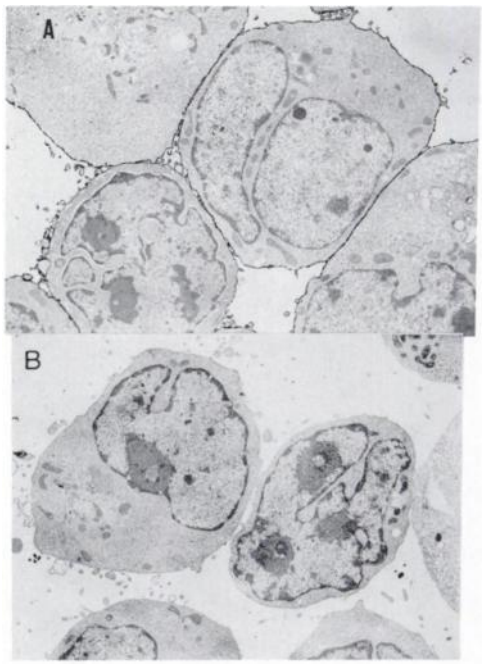

Fig. 7. Immunoelectron micrographs of 2780 cells (*A*) and A2780 cells (*B*). Note that P-glycoprotein is present on 2780 cells but is almost entirely lacking on A2780 cells. × 5000.

DISCUSSION

The present study revealed that: (*a*) P-glycoprotein recognized by MRK 16 MAb was expressed significantly strongly in adult adrenal, kidney, and placental tissue and in some cases of untreated lung and breast carcinoma; (*b*) P-glycoprotein was not recognizable in fetal and neonatal adrenals; and (*c*) ultrastructurally, the P-glycoprotein recognized by MRK 16 MAb was distributed densely and homogeneously in K-562/ADM and 2780^{AD} cells.

From these results, there are three points to be commented upon. (*a*) Our immunostaining data strongly imply that P-glycoprotein plays a physiological role in the adult adrenal, kidney, and placenta. This may be especially important in the adult adrenal, and the level of P-glycoprotein may increase with age, since no P-glycoprotein was found in immature adrenal. It is reported that the *mdr* 1 gene encoding P-glycoprotein is mainly expressed in the adrenal medulla (16). As our data showed that P-glycoprotein exists in both the adrenal cortex and medulla, we are now attempting to identify the P-glycoprotein-expressing cells in the adrenals.[4] Although it has been reported that the liver, lung, and large and small intestines also express intermediate levels of *mdr* 1 mRNA, we were unable to observe any increase in P-glycoprotein at the protein level. Further study will be required in order to determine why such a disparity exists between the levels of P-glycoprotein and its mRNA in tissues.

(*b*) As we found that the P-glycoprotein was recognizable at a high level in untreated lung and breast cancers, increased expression of P-glycoprotein may be involved in the natural resistance of tumors to anticancer agents. The two patients who possessed P-glycoprotein in their cancerous tissues are currently still alive, following complete excision of the malignant tissues. Although we were unable to use relatively fresh surgical specimens from cancer patients who had been treated with anticancer agents, we are accumulating more data in order to determine whether there may be an elevated level of P-glycoprotein in leukemic cells from patients treated with Adriamycin, as revealed by Spectrum III (Orthodiagnostics).[4] Extensive studies will be required, using cancerous tissues from treated patients.

Finally, the EGF receptor has an apparent similarity to the P-glycoprotein reported in this paper with regard to molecular weight and its glycoprotein nature. We have performed immunoperoxidase staining of MRK 16 MAb-positive surgical specimens such as adrenal, placenta, and lung cancer, utilizing an anti-EGF receptor murine MAb (528 MAb) provided by Dr. T. Kawamoto (17) and found that none was stained positively.[3]

From our present data, it is suggested that MRK 16 MAb may have two potentially useful clinical applications. One is that the MAb, either by itself or in combination with toxins or radioisotopes, may be used for the selective killing of cancer cells *ex vivo* containing high levels of P-glycoprotein. The other is that the MAb may be useful for detecting the degree of multidrug resistance in various types of malignancy *in vitro*.

[4] Manuscript in preparation.

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