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Further Characterization of the Human Adrenal-derived P-Glycoprotein Recognized by Monoclonal Antibody MRK 16 Reacting with Only Human P-Glycoprotein

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This paper describes further characterization of the 170–180-kDa glycoprotein (P-glycoprotein) recognized by the monoclonal antibody MRK 16 in the human adrenal. By electron microscopy, P-glycoprotein was observed in the adrenal cell membranes. However, MRK 16-defined P-glycoprotein was not found in cow, pig, horse, monkey or rabbit adrenal, indicating that MRK 16 recognizes the non-homologous part of P-glycoprotein of various species. Eleven out of 16 adrenal tumors including 4 cases of primary aldosteronism and 7 cases of Cushing syndrome were intensely stained with MRK 16, whereas pheochromocytoma, non-functioning adrenocortical adenoma with no associated increase of serum adrenal-derived hormones and myolipoma of the adrenal were not. Finally, P-glycoprotein-MRK 16-protein A-Sepharose complex derived from human adrenal possessed marked ATPase activity. Taken together, these data suggest that P-glycoprotein may play a physiological role in the human adrenal.

Key words: ATPase activity — P-glycoprotein — Adrenal — Adrenal tumors

It has been shown recently that the adriamycin-resistant K562 cell line, K562/ADM,[6] possesses a 170–180 kDa P-glycoprotein in the cell membrane, and displays multidrug resistance against Vinca alkaloids, etoposide and cholchicine.[1,2] This P-glycoprotein probably plays an important role in drug efflux.[3] Furthermore, this P-glycoprotein is known to be highly expressed in other organs including adrenal, kidney and placenta, and also in some untreated lung and breast cancers.[4] It is of particular interest that P-glycoprotein is present in the adrenal, irrespective of administration of anti-cancer agents, and that the amount of P-glycoprotein increases with age.[5]

The present study focused on immunoelectron microscopical localization of P-glycoprotein in the human adrenal, the expression of P-glycoprotein defined by monoclonal antibody MRK 16 in the adrenals of other species, the level of expression of P-glycoprotein in adrenocortical adenomas and the ATPase activity of P-glycoprotein in the human adrenal as a physiological function.

MATERIALS AND METHODS

Cell line and tissues The adriamycin-resistant cell line K562 (K562/ADM) was originally established in the laboratory of one of the authors.[1,6] It was grown in RPMI 1640 medium containing 10%fetal calf serum and gentamicin sulfate (20 μg/ml) and routinely passaged twice weekly.

For the immunohistochemical study, 16 surgical specimens of adrenal tumor, myolipoma of the adrenal and normal human adrenal (autopsy case) were obtained from the Third Department of Internal Medicine, Nara Medical University, Nara. The clinical profiles of the patients from which the specimens were taken are given in Table I. Horse, pig, cow, rabbit and monkey adrenals were supplied by the Department of Virology, National Institute of Health, Tokyo. Tissues for frozen-section studies were immediately snap-frozen in liquid nitrogen and stored at -70°C until sectioning.

Monoclonal antibodies The methods used for antigen preparation, immunization, cell fusion, cloning, and serological characterization of the monoclonal antibody (MAb) MRK 16 isotype have been described previously

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[6] Abbreviations used in the text are as follows: K562/ADM, adriamycin-resistant K562; MAb, monoclonal antibody; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PLP, periodate-lysine-paraformaldehyde; kDa, kilodaltons; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; ABC-PO, avidin-biotin-peroxidase complex.

Table I. Clinical Profiles of the Subjects Used in This Study and Adrenal Reactivity to MRK16[a]

Case No.	Sex	Clinicopathological diagnosis	Reactivity		Immuno-precipitation
			MRK 16	C219	
1	F	Adrenal virilizing syndrome	+[b]	+[b]	+
2	F	Cortical adenoma of the adrenal (Cushing syndrome)	+	+	+
3	F	Non-functioning adenoma of the adrenal cortex	−	−	−
4	F	Cortical adenoma of the adrenal (Cushing syndrome)	+	+	+
5	F	Cortical adenoma of the adrenal (Cushing syndrome)	+	+	+
6	M	Myolipoma of the adrenal	−	−	−
7	F	Cortical adenoma of the adrenal (Cushing syndrome)	+	+	+
8	F	Cortical adenoma of the adrenal (Primary aldosteronism)	+	+	+
9	F	Cortical adenoma of the adrenal (Cushing syndrome)	+	+	+
10	F	Cortical adenoma of the adrenal (Cushing syndrome)	+	+	+
11	F	Cortical adenoma of the adrenal (Cushing syndrome)	+	+	+
12	F	Cortical adenoma of the adrenal (Primary aldosteronism)	+	+	+
13	F	Cortical adenoma of the adrenal (Primary aldosteronism)	+	+	+
14	M	Pheochromocytoma	−	−	−
15	M	Myolipoma of the adrenal	−	−	−
16	M	Cortical adenoma of the adrenal (Primary aldosteronism)	+	+	+

a) All cases were untreated.
b) Intensity of immunohistochemistry: +, positive; −, negative.

in detail.[7] Mouse MAb C219 was used for the present study as a purified immunoglobulin preparation at 15 μg/ml (Centocor, Malvern, PA).[8]

Biotinylation of MAbs MRK 16 and C219 and immunohistochemistry Biotinylated MAbs MRK 16 and C219 were prepared according to the method of Guesdon *et al.*[9] Briefly, 1 ml of a solution of 0.1 *N* $NaHCO_3$ containing 1 mg/ml MRK 16 MAb was mixed with 60 μl of biotinyl-N-hydroxysuccinimide (BNHS, 1 mg/ml DMSO) (Sigma). The reaction mixture was incubated at room temperature for 4 h and then dialyzed for 24 h at 4°C against several changes of PBS. The solution was adjusted to 10 μg/ml PBS prior to use.

Frozen sections, 4 μm in thickness, of the adrenal tumors were prepared for avidin-biotin-peroxidase complex (ABC-PO) staining as described elsewhere.[10]

Biotinylated MRK 16 MAb was examined for its reactivity with horse, pig, cow, rabbit and monkey adrenals. Briefly, after 30-min blocking with 5% bovine serum albumin at room temperature, biotinylated MRK 16 (10 μg/ml) was reacted with sections of the adrenals at the same temperature for 30 min. The sections were washed carefully with PBS three times, then ABC-PO solution was placed on them at room temperature for 30 min (according to the instruction sheet issued by Vector Labs., Burlingame, CA). After three washings with PBS, the sections were developed with 100 μl of 3% H_2O_2 and 200 μg/ml diaminobenzidine for 5 min and examined with a light microscope (Olympus Optical Co., Tokyo).

Immunoelectron microscopic study To examine the localization of the P-glycoprotein recognized by MRK 16 in the human adrenal, an immunoelectron microscopical study was performed as described previously.[4]

***In vitro* phosphorylation and immunoprecipitation** *In vitro* phosphorylation was carried out essentially as described previously.[5, 11, 12]

Preparation of crude membrane fractions K562/ADM, adenoma and adrenal tissue were homogenized, sonicated for 15 min at 4°C and suspended in hypotonic lysis buffer (10 m*M* Tris-HCl, pH 7.4, 10 m*M* NaCl, 1.5 m*M* $MgCl_2$, 1 m*M* dithiothreitol). The suspensions were then incubated for 15 min in an ice-bath. The nuclei were removed by centrifugation at 400*g* for 10 min. The enucleated cell homogenate was then spun at 22,000*g* for 30 min and the pellet was used as a crude membrane fraction.

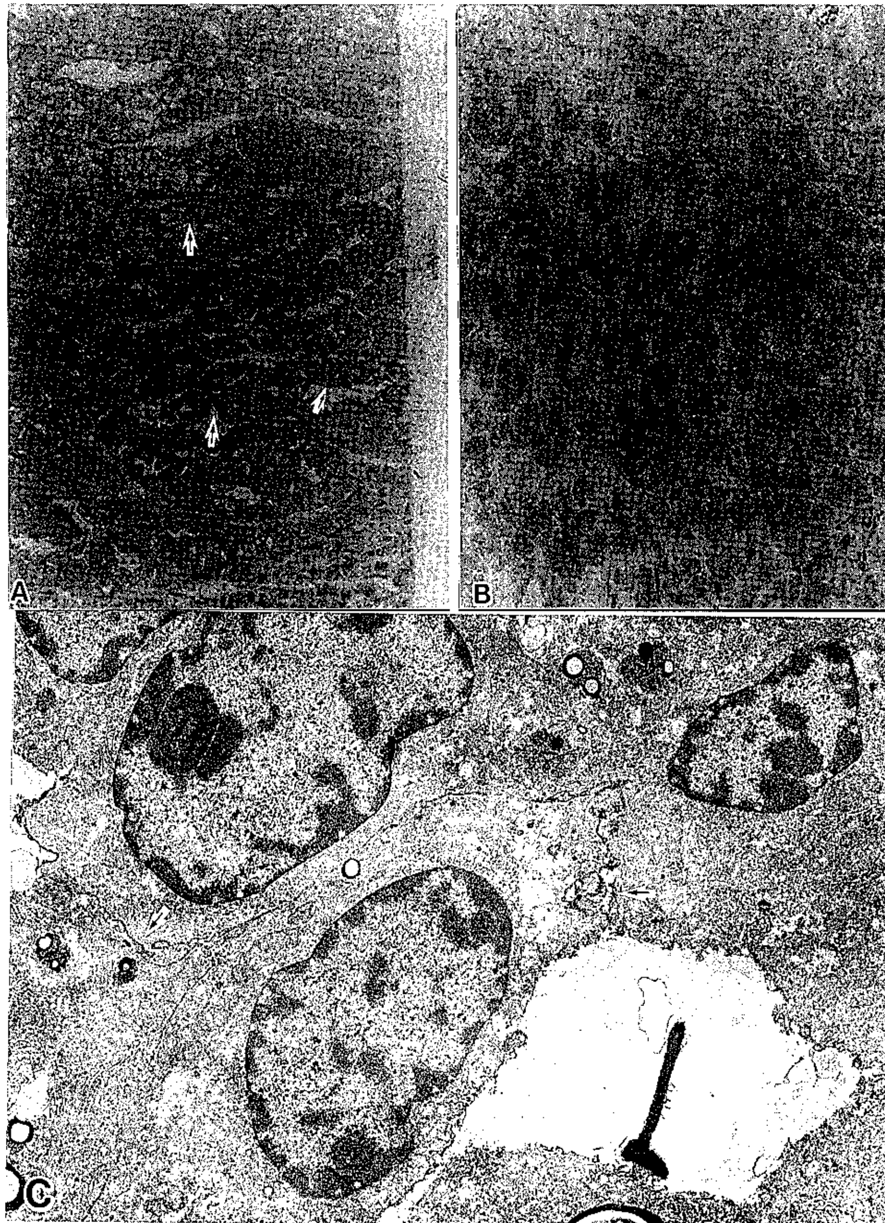

Fig. 1. Ultrastructural localization of MRK 16 reactivity in the human adrenal cortex. A: Semi-thin section of the human adrenal cortex. ×600. MRK 16 MAb-defined P-glycoprotein is present on the cell membranes (arrows). B: Semi-thin section of the human adrenal cortex. ×600. Negative control (non-immune mouse serum instead of MRK 16 MAb). C: Immunoelectron microscopical localization of P-glycoprotein from the human adrenal cortex (arrows). ×7,500.

Table II. Reactivity of Biotinylated MAb MRK 16 with Adrenals of Various Species

Adrenal tissue	Reactivity[a]		Immunoprecipitation with MRK 16 MAb[b]
	Biotinylated MRK 16 MAb	C219	
Human	+	+	+
Monkey	−	+	−
Horse	−	+	−
Pig	−	+	−
Cow	−	+	−
Rabbit	−	+	−

a) Intensity of immunoperoxidase staining (ABC-PO method) is classified as follows: −, negative; +, almost all the tissue stained with MRK 16 or C219 MAb.
b) +: Presence of P-glycoprotein immunoprecipitated with MRK 16 MAb. −: Absence of P-glycoprotein immunoprecipitated with MRK 16 MAb.

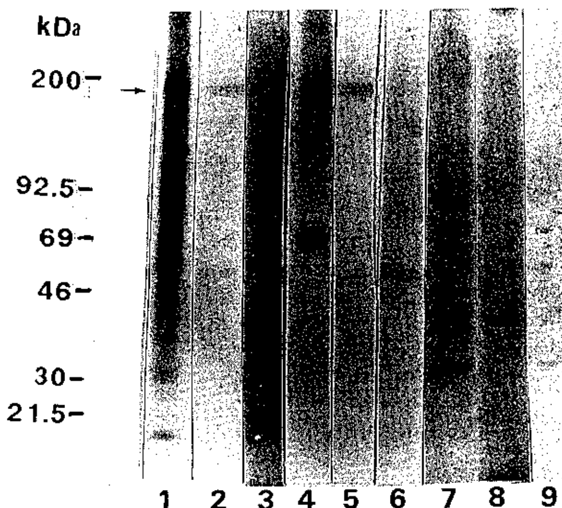

Fig. 2. *In vitro* phosphorylation of adrenals of various species and in cortical adenomas of the adrenal. Lane 1, Phosphorylation pattern of K562/ADM precipitated with MRK 16; lane 2, phosphorylation pattern of human adrenal precipitated with MRK 16; lane 3, phosphorylation pattern of human adrenal precipitated with non-immune mouse sera instead of MRK 16 MAb; lane 4, cortical adenoma of the adrenal (case 7); lane 5, cortical adenoma of the adrenal (case 12); lane 6, non-functioning adenoma (case 3); lane 7, monkey adrenal; lane 8, horse adrenal; lane 9, pig adrenal. An arrow indicates the band of P-glycoprotein.

Preparation of P-glycoprotein-antibody-protein A-Sepharose complexes Membrane preparations from K562/ADM, adenoma and adrenal tissue were solubilized in lysis buffer (50 m*M* Tris-HCl, pH 8.0, 150 m*M* NH_4Cl, 2 m*M* $MgCl_2$, and 1% CHAPS) for 30 min in an ice-bath. Immunoprecipitation was carried out by incubating the membrane lysates (1.0 mg protein/ml) with 25 μg/ml MRK 16 for 2 h at 4°C. Then 100 μl of protein A-Sepharose CL-4B suspension (20% by volume in lysis buffer) was added to 1 ml of each membrane lysate. After incubation for 30 min, the precipitate was washed five times with the reaction buffer (50 m*M* Tris-HCl, pH 7.4, 150 m*M* NH_4Cl, and 0.1% CHAPS). The resulting P-glycoprotein-antibody-protein A-Sepharose complexes were used for assay of ATPase. The amount of the protein immobilized on the carrier was estimated by SDS-PAGE analysis of the complex followed by Coomassie blue staining.

ATPase assay The hydrolysis of ATP by P-glycoprotein was assayed by measuring the formation of ADP from [α-^{32}P]ATP as described previously,[13] with some modifications. Standard reaction mixtures (100 μl) contained 50 m*M* Tris-HCl (pH 7.4), 150 m*M* NH_4Cl, 2 m*M* $MgCl_2$, 0.1% CHAPS, [α-^{32}P]ATP at 150 μ*M*, and P-glycoprotein-antibody-protein A-Sepharose complex (20 μl wet gel volume). The preparation was mixed for about 5 s then left at 30°C for 20 min unless otherwise indicated. The reaction was stopped by adding 25 μl of 50 m*M* EDTA, 2.5% SDS, 25 m*M* ATP and 25 m*M* ADP. The immobilized enzyme was centrifuged off at 10,000*g* for 10 s. Samples were spotted on polyethyleneimine cellulose thin-layer plates (20×20 cm, Macherey-Nagel) and developed in 0.5 *M* potassium phosphate buffer, pH 3.4. The ADP-containing spots were visualized using UV light (253.6 nm) and cut out, and the amount of radioactivity (Cerenkov) in each was determined using a liquid scintillation counter. The ATPase activity was expressed as the amount of specifically hydrolyzed ATP.

RESULTS

Ultrastructural localization of P-glycoprotein recognized by MRK 16 MAb in the human adrenal As shown in Fig. 1, P-glycoprotein was present in the cell membranes of the adrenal cortex. Three layers (zona glomerulosa, zona

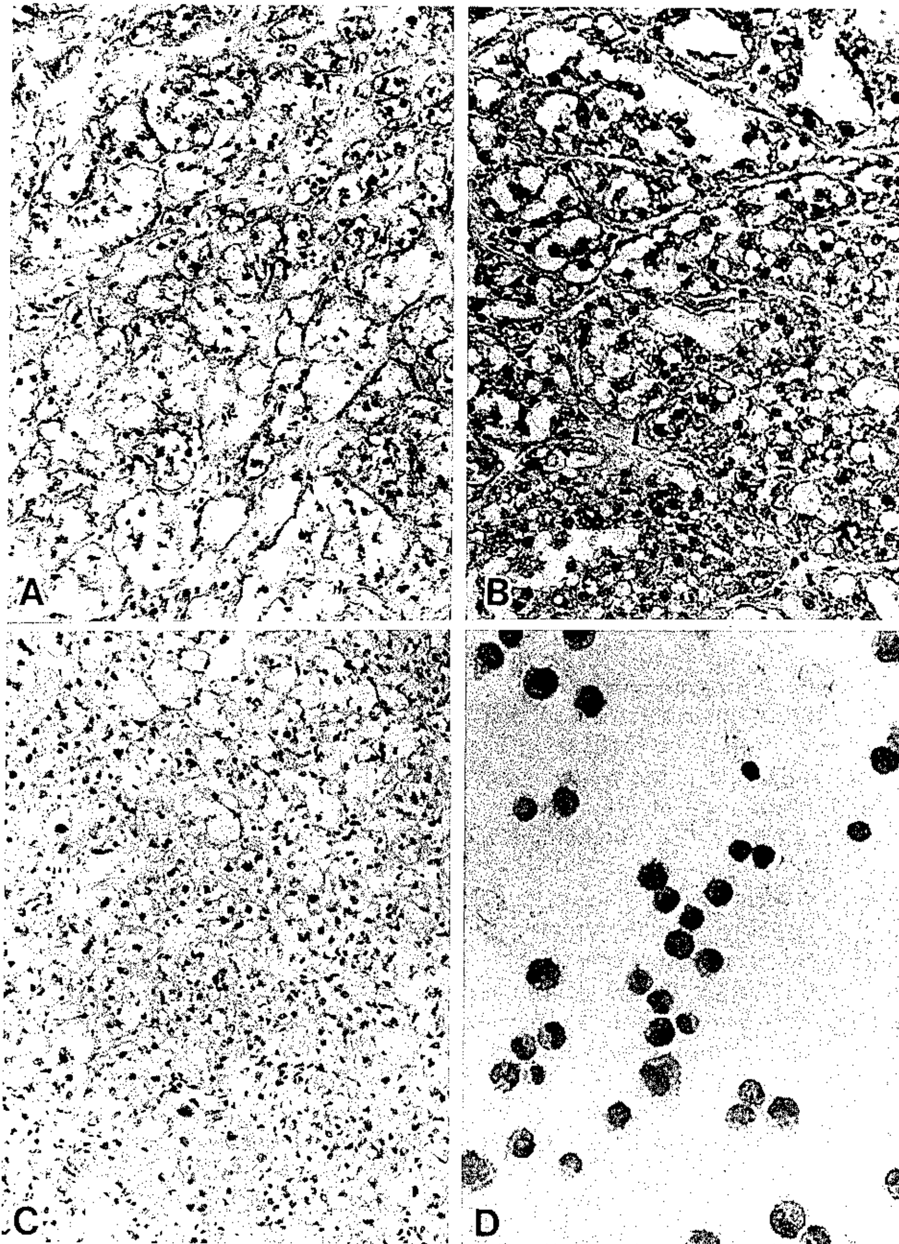

Fig. 3. Reactivity of cortical adenomas of the adrenal with MAb MRK 16. A: Case 11 (Cushing syndrome). B: Case 13 (primary aldosteronism). C: Case 3 (non-functioning adenoma). D: K562/ADM (positive control).

fasciculata, and zona reticularis) possessed P-glycoprotein.

Level of expression of P-glycoprotein recognized by MRK 16 MAb in the adrenal of other species Next, we investigated whether MRK 16 MAb recognized P-glycoprotein in the adrenals of other species (horse, pig, cow, rabbit and monkey). As shown in Table II, MRK 16 did not react with P-glycoprotein in the adrenals of these animals. However, C219 MAb did react with these adrenal specimens. This finding was further confirmed by data obtained in immunoprecipitation tests (Fig. 2).

Reactivities of MRK 16 and C219 with adrenal tumors As summarized in Table I, 11 out of 16 adrenal tumors (Cushing syndrome, primary aldosteronism and viriliz-

ing adenoma) reacted strongly with MRK 16 as well as C219. However, pheochromocytoma, non-functioning adenoma and myolipoma of the adrenal showed reaction. This finding was also confirmed by immunoprecipitation data (Figs. 2 and 3).

ATPase activity of P-glycoprotein derived from human adrenal As it is known that P-glycoprotein from adriamycin-resistant K562 (K562/ADM) has ATPase activity, we examined the ATPase activity of P-glycoprotein in the human adrenal. As shown in Fig. 4, human adrenal P-glycoprotein possessed ATPase activity, but at a level lower than that of P-glycoprotein from K562/ADM. The P-glycoprotein from K562 possessed slight but significant ATPase activity because K562 cells reacted weakly with MRK 16 as assessed by immunocytochemistry (Fig. 4).

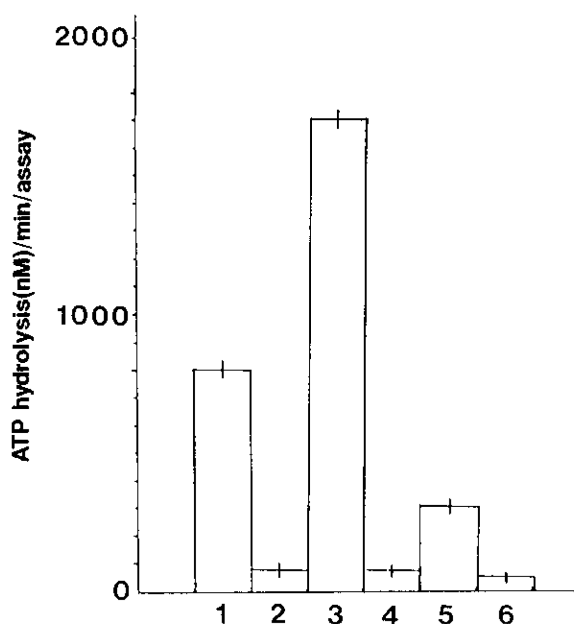

Fig. 4. ATPase activity of P-glycoprotein-MRK 16 MAb-protein A-Sepharose complex from the human adrenal. 1. Adrenal. 2. Adrenal reacted with non-immune mouse serum. 3. K562/ADM. 4. K562/ADM reacted with non-immune mouse serum. 5. K562. 6. K562 reacted with non-immune mouse serum.

DISCUSSION

The present study revealed four findings. First, the human adrenal possesses P-glycoprotein, which is present in the cell membranes of cells in three layers (zona glomerulosa, zona fasciculata and zona reticularis).

Second, MAb MRK 16 does not react with adrenal tissue from pig, horse, cow, rabbit or monkey. This indicates that MRK 16 recognized a non-homologous part of P-glycoprotein from adrenals of other species, whereas C219 MAb did react with these adrenal specimens.

Third, MRK 16 reacted with cortical adenomas of human adrenals secreting testosterone, aldosterone and corticosteroid, suggesting that these adenomas possess P-glycoprotein. MRK 16 did not react with pheochromocytoma, non-functioning cortical adenoma (adrenal-derived hormones in the serum were not increased) or myolipoma of the adrenal. Therefore, it is speculated that P-glycoprotein transports hormones or hormone metabolites through the plasma membranes of adrenal cortical cells.

Finally, P-glycoprotein-MRK 16-protein A-Sepharose complex from human adrenal possesses ATPase activity. However, P-glycoprotein-C219-protein A-Sepharose complex lacked ATPase activity (data not shown). This difference in ATPase activity may reflect the different P-glycoprotein domains recognized by these antibodies (personal communication with Dr. V. Ling). It has been shown recently that P-glycoprotein from multidrug-resistant cells has ATPase activity, implying that the ATPase may play a role in the efflux of certain anticancer drugs.[14] This ATPase may also be involved in the efflux of adrenal hormones and/or their metabolites, although further verification of this possibility will be required. As it has been reported that P-glycoprotein from uterine epithelia during pregnancy binds to progesterone,[15] it would be of great interest to examine the binding of P-glycoprotein to adrenal hormones.

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REFERENCES

1) Tsuruo, T., Iida-Saito, H., Kawabata, H., Oh-hara, T., Hamada, H. and Utakoji, T. Characteristics of resistance to adriamycin in human myelogenous leukemia K562 resistant to adriamycin and in isolated clones. *Jpn. J. Cancer Res.*, **77**, 682–692 (1986).
2) Pastan, I. and Gottesman, M. Multiple-drug resistance in human cancer. *N. Eng. J. Med.*, **316**, 1388–1393 (1987).
3) Cornwell, M. M., Safa, A. R., Felsted, R. L., Gottesman,

M. M. and Pastan, I. Membrane vesicles from multidrug-resistant cancer cells contain a specific 150- to 170-kDa protein detected by photoaffinity labeling. *Proc. Natl. Acad. Sci. USA*, **8**, 3847–3850 (1986).

4) Sugawara, I., Kataoka, I., Morishita, Y., Hamada, H., Tsuruo, T., Itoyama, S. and Mori, S. Tissue distribution of P-glycoprotein encoded by a multidrug-resistant gene as revealed by a monoclonal antibody, MRK 16. *Cancer Res.*, **48**, 1926–1929 (1988).
5) Sugawara, I., Nakahama, M., Hamada, H., Tsuruo, T. and Mori, S. Apparent stronger expression in the human adrenal cortex than in the human adrenal medulla of Mr 170,000–180,000 P-glycoprotein. *Cancer Res.*, **48**, 4611–4614 (1988).
6) Sugimoto, Y. and Tsuruo, T. DNA-mediated transfer and cloning of a human multidrug-resistant gene of adriamycin-resistant mylogenous leukemia K562. *Cancer Res.*, **47**, 2620–2625 (1987).
7) Hamada, H. and Tsuruo, T. Functional role for the 170- to 180-kDa glycoprotein specific to drug-resistant tumor cells as revealed by monoclonal antibodies. *Proc. Natl. Acad. Sci. USA*, **83**, 7785–7789 (1986).
8) Kartner, N., Evernden-Porelle, D., Bradley, G. and Ling, V. Detection of P-glycoprotein in multidrug-resistant cell lines by monoclonal antibodies. *Nature*, **316**, 820–823 (1985).
9) Guesdon, J-L., Ternynck, T. and Avrameas, S. The use of avidin-biotin interaction in immunoenzymatic techniques. *J. Histochem. Cytochem.*, **27**, 1131–1139 (1979).
10) Sugawara, I., Ohkochi, E., Hamada, H., Tsuruo, T. and Mori, S. Cellular and tissue distribution of MRK20 murine monoclonal antibody-defined 85-kDa protein in adriamycin-resistant cancer cell lines. *Jpn. J. Cancer Res.*, **79**, 1101–1110 (1988).
11) Hamada, H. and Tsuruo, T. Growth inhibition of multi-drug-resistant cells by monoclonal antibodies against P-glycoprotein. *In* "Molecular and Cellular Biology of Multidrug Resistance in Tumor Cells," ed. I. Roninson, in press (1988). Plenum Press, New York.
12) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685 (1970).
13) Hamada, H. and Tsuruo, T. Purification of the 170- to 180-kilodalton membrane glycoprotein associated with multidrug resistance. *J. Biol. Chem.*, **263**, 1454–1458 (1988).
14) Hamada, H. and Tsuruo, T. Characterization of the ATPase activity of the Mr 170,000 to 180,000 membrane glycoprotein (P-glycoprotein) associated with multidrug resistance in K562/ADM cells. *Cancer Res.*, **48**, 4926–4932 (1988).
15) Yang, C-P. H., DePinho, S. G., Greenberger, L. M., Arceci, R. J., and Horwitz, S. B. Progesterone interacts with P-glycoprotein in multidrug-resistant cells and in the endometrium of gravid uterus. *J. Biol. Chem.* (1988), in press.