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Publication date
1997

Published in
Investigative Ophthalmology & Visual Science

[Link to publication](#)

Citation for published version (APA):

van der Pol, J. P., Blom, D. J. R., Flens, M. J., Luyten, G. P. M., de Waard-Siebenga, I., Koornneef, L., Scheper, R. J., & Jager, M. J. (1997). Multidrug resistance: related proteins in primary choroidal melanoma and in vitro cell lines. *Investigative Ophthalmology & Visual Science*, 38, 2523-2530.

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Multidrug Resistance–Related Proteins in Primary Choroidal Melanomas and In Vitro Cell Lines

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Purpose. Metastatic uveal melanoma is strongly resistant to chemotherapy, and multidrug resistance (MDR) may be involved. To investigate the role of MDR, the presence of the MDR-associated proteins P-glycoprotein (Pgp), MRP, and lung resistance protein (LRP) was determined on primary choroidal melanomas and cell lines.

Methods. A panel of primary choroidal melanomas was examined for the presence of MDR-associated proteins by immunohistochemical analysis. In cell lines established from four primary choroidal melanomas and one metastatic choroidal melanoma, the expression of MDR-associated proteins was determined with monoclonal antibodies in cytospin preparations and flow cytometry. In addition, the functional capacities of transporter proteins Pgp and MRP as adenosine triphosphate-driven efflux pumps were determined by measuring the cellular accumulation and efflux of the fluorescent dyes rhodamine 123 and calcein-AM, with and without the presence of specific pump inhibitors PSC833 and probenecid.

Results. Low levels of Pgp and MRP were detected in most primary tumors and in some cell lines. Measurable transporter function of Pgp could be determined in cell line OCM-1. Lung-resistance protein was present in all primary tumors and cell lines and showed high expression levels.

Conclusions. This study revealed the involvement of LRP and at least a minor role of Pgp and MRP in chemoresistance of choroidal melanoma. Compared with cutaneous melanomas, uveal melanomas appear to express slightly higher levels of Pgp. These findings provide insights into the drug-resistant phenotype of this disease and can aid in the design of therapeutic protocols. *Invest Ophthalmol Vis Sci.* 1997;38:2523–2530.

The prognosis of patients with metastatic choroidal melanoma is extremely poor. Once the diagnosis of hepatic metastasis has been made, the median survival time is 2 to 7 months.^{1,2} Immunotherapy and modified regimens of drug administration are under investigation, but the resistance of this disease to chemotherapy is one of the major reasons for the current absence of any effective treatment of metastases.² Cytotoxic

agents are successful in many malignancies (e.g., testicular cancer, leukemia), but these agents have had little success in metastatic uveal melanoma.³ Tumor cells may be resistant to a specific drug or a group of drugs with structural or functional similarities, and there is increasing evidence that several mechanisms allow tumor cells to develop resistance to a variety of drugs that differ in structure and function.⁴ This is called multidrug resistance (MDR). The most frequently described mechanism in MDR involves overexpression of a 170-kDa plasma membrane protein, P-glycoprotein (Pgp), which is encoded by the human MDR1 gene.^{5,6} This protein acts as an adenosine triphosphate-driven drug efflux pump and has been shown to cause “classical MDR,” conferring resistance to anthracyclines, epipodophyllotoxins, actinomycin D, vinca alkaloids, and some alkylating agents.^{6–8} The presence of Pgp in normal tissues such as colon, liver, and kidney and at the blood–brain barrier suggests

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Supported by the Haagsch Oogheelkundig Fonds and Stichting Blinden-Penning and Edmond en Marianne Blaauw Fonds.
Submitted for publication February 24, 1997; revised July 14, 1997; accepted July 14, 1997.

Proprietary interest category: N.

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that it has a physiological role in the defense against xenobiotics.⁹ Increased expression of Pgp has been detected in various tumors arising from tissues that normally express Pgp, as well as in tumors originating from cells that do not (e.g., myelomas, sarcomas).⁹ In some cancers (e.g., childhood malignancies, multiple myeloma, some adult leukemias), clinically relevant correlations have been established between Pgp expression and the results of chemotherapy.¹⁰

Several lipophilic compounds have been identified that can reverse the Pgp-mediated resistance to chemotherapy.^{11,12} Reversal compounds (e.g., verapamil, cyclosporin) have been used with some success to overcome drug resistance.^{13,14} A second MDR-related protein, MRP, was identified in cell lines showing drug resistance without expression of Pgp.¹⁵⁻¹⁷ Like Pgp, MRP belongs to the adenosine triphosphate-binding cassette superfamily of membrane transport proteins and also causes resistance by acting as a molecular efflux pump.^{18,19} The drug-resistance pattern conferred by the MRP gene is similar but not identical to that of Pgp.^{17,19}

Recently, a third MDR-associated protein, lung resistance protein (LRP), was identified.²⁰ This protein forms the major component of the newly described cellular organelles named vaults.²¹ Vaults are distributed mainly through the cytoplasm, and a few of them are localized at the nuclear membrane and nuclear pore complexes. It has been suggested that vaults play a role in drug resistance by regulating both the nucleocytoplasmic transport and the cytoplasmic redistribution of drugs.^{21,22} Lung resistance protein is a strong predictor of poor response to chemotherapy and prognosis in acute myeloid leukemia and ovarian cancer.^{23,24}

To understand the mechanisms involved in the high resistance of metastatic choroidal melanoma to chemotherapeutic agents, we investigated the expression of Pgp, MRP, and LRP on sections of choroidal melanomas and on uveal melanoma cell lines. The functional capacities of transporter proteins were determined in functional assays.

MATERIALS AND METHODS

Primary choroidal melanomas were examined for MDR-associated proteins using immunoperoxidase staining of fresh-frozen tissue. Cell lines isolated from primary choroidal melanomas and one metastasis from choroidal melanoma were studied using immunohistochemistry and flow cytometry. Because the degree of immunostaining of antigens that are associated with functional proteins does not necessarily represent the number of active transporter proteins (antigens on nonfunctional proteins are stained as well), functional assays were conducted for the presence of Pgp

and MRP. True functional capacities of transporter proteins Pgp and MDR as adenosine triphosphate-driven efflux pumps were determined by measuring cellular accumulation and efflux of fluorescent dyes with and without the presence of specific pump inhibitors. For LRP, no inhibitor is known, so no functional assay was available.

Tissue Specimens

Specimens from primary choroidal melanomas were obtained from storage banks of the departments of ophthalmology of the university hospitals of Amsterdam and Leiden, The Netherlands. All 12 eyes were enucleated without prior therapy. Part of the tumor was snap-frozen in OCT compound (Ames Co., Div. of Miles Laboratories, Elkhart, IN) and stored at -70°C . The remainder of the eye was fixed in formalin and embedded in paraffin or celloidin (Leiden) for histopathologic analysis. All material used in this study was obtained from routinely stored specimens and did not interfere in any way with diagnostic or therapeutic procedures for patients.

Immunohistochemical Analysis

Cryostat sections of tissue samples were cut $4\ \mu\text{m}$ thick, air dried overnight, and fixed in acetone for 10 minutes at room temperature. The slides were incubated for 20 minutes with normal rabbit serum (1:50; DAKO, Copenhagen, Denmark) followed by monoclonal antibodies (C219²⁵ [1:10] and JSB-1,²⁶ MRPr1,²⁷ MRPM6,²⁷ LRP-56,²⁰ and LMR-5 [all 1:20]) and conjugates diluted in phosphate-buffered saline and bovine serum albumin. Isotype-matched negative controls were used.

Staining was done using an indirect immunoperoxidase technique with peroxidase-conjugated rabbit antirat or antimouse immunoglobulin G (IgG) (1:25; DAKO). Monoclonal antibody (MAb) binding was detected using biotinylated rabbit antimouse IgG (1:150; DAKO) or biotinylated rabbit antirat IgG (1:100; DAKO) and streptavidin conjugated to horseradish peroxidase (1:500; Zymed, San Francisco, CA). Bound peroxidase was developed with 4 mg (wt/vol) 3-amino-9 ethylcarbazole and 0.02% H_2O_2 in 0.1 M NaAc (pH 5.0). The slides were counterstained with hematoxylin and mounted. Immunoreactivity was scored semiquantitatively in four categories: no, weak, positive, and strongly positive staining. All slides were scored by two observers. No major discrepancies (>one category) occurred, and smaller discrepancies were resolved after parallel examination of the slides.

Melanoma Cell Lines

Five human choroidal melanoma cell lines (92-1,²⁸ OMM-1,²⁹ OCM-1,³⁰ EOM-3,²⁹ and Mel 202³¹) were used. All cell lines were established from primary cho-

TABLE 1. Staining of Multidrug Resistant-associated Proteins in a Panel of Primary Choroidal Melanomas

Tumor	Cell Type	Largest Diameter	P-Glycoprotein	MRP	LRP
1	Epithelioid	17	●	●	●●
2	Spindle	ND	○	●	●
3	Mixed	17.5	○	○	○
4	Spindle	12	●	●	●
5	Epithelioid	15	●	●	●
6	Mixed	25	○	●	●●
7	Mixed	10	●	●	●
8	Spindle	15	●	●●	●●
9	Spindle	11	○	●	●
10	Mixed	13	○	●	●
11	Spindle	23	○	●●	●
12	Mixed	12	○	●	●●

Staining intensities were scored according to the following scale: ○ = no staining; ● = weak staining; ● = positive staining; ●● = strongly positive staining. ND = not done, could not be determined; LRP = lung resistance protein.

roidal melanomas except OMM-1, which originated from a choroidal melanoma metastasis. Cell lines 92-1, EOM-3, and Mel 202 were used at approximately 60 passages and cell lines OMM-1 and OCM-1 at approximately 50 passages. In vitro doubling time of cell line EOM-3 was approximately 96 hours, cell lines 92-1 and Mel 202 doubled in 54 hours, and OMM-1 and OCM-1 were the fastest-growing cells, with doubling times of 50 hours.

Cell line 92-1 was established in the laboratory of the Department of Ophthalmology, Leiden University Hospital, The Netherlands; cell lines OMM-1 and EOM-3 came from the Department of Ophthalmology, Erasmus University, Rotterdam, The Netherlands. Cell line OCM-1 was a generous gift from J. Kan-Mitchell (University of California at San Diego). Cell line Mel 202 was kindly provided by B. Ksander (Schepens Eye Research Institute, Harvard Medical School, Boston, MA).

Flow Cytometric Analysis

In flow cytometry analysis, cells were permeabilized with a 10% lysing solution (Solution G; Becton Dickinson, San Jose, CA) in distilled water for 15 minutes and incubated for 15 minutes at 4°C in phosphate-buffered saline-bovine serum albumin containing 1% normal goat serum for measurements with MAbs C219, MRPr1, MRPr6, LRP-56, and LMR-5 (all stain intracellular antigens). Cell lines were not permeabilized for determination of Pgp with MAb MRK-16 (stains extracellular epitopes of Pgp).³² Cells (10⁵) were then incubated for 1 hour at 4°C in 500 μl phosphate-buffered saline-bovine serum albumin containing one of the Pgp, MRP, or LRP MAbs, or rat or mouse isotype-matched control MAbs. Antibody binding was detected with fluorescein isothiocyanate-

labeled goat antirat or antimouse immunoglobulins (1:100; DAKO). Fluorescence was analyzed on a FACScan II (Becton Dickinson). Results are expressed as the ratio of the two mean fluorescences of specifically stained cells and controls. Controls were cell line GLC4, a drug-sensitive small cell lung cancer cell line expressing low levels of MRP and LRP, and the corresponding subline GLC4/ADR, which overexpresses MRP and LRP and is strongly drug resistant.³³ For analysis of Pgp expression, cell lines KB3-1 (low Pgp expression; sensitive to doxorubicin) and KB8-5 (overexpression of Pgp; twofold resistance to doxorubicin) served as controls.³⁴

Immunocytochemical Analysis

Cytospin preparations of tumor cell lines were air-dried and fixed for 10 minutes in acetone. The slides were incubated for 1 hour with MAb diluted in phosphate-buffered saline containing 1% bovine serum albumin. 4E3³⁵ (Signet, Dedham, MA) was diluted 1:10 and JSB-1, MRPr1, MRPr6, LRP-56, and LMR-5 were used at dilutions of 1:20. Isotype-matched negative controls were used. Monoclonal antibody binding was detected using biotinylated rabbit antirat IgG (1:100; DAKO) or antimouse IgG (1:150; DAKO) or streptavidin-conjugated horseradish peroxidase (1:500; Zymed). Bound peroxidase was developed with 4 mg (wt/vol) amino-ethyl-carbazole and 0.02% H₂O₂ in 0.1 M NaAc (pH 5.0), counterstained with hematoxylin, and mounted with Aquamount (Gurr, BDH Chemicals, Poole, UK). Immunoreactivity was determined as in immunohistochemical analysis.

Functional Assay of Cellular Drug Accumulation and Efflux

The transporter function of Pgp-associated proteins in cell lines was determined by flow cytometry, using

rhodamine 123 (200 ng/ml⁻¹; Sigma, St. Louis, MO) and calcein-AM (0.5 μM; Molecular Probes, Eugene, OR) as substrates and PSC833 (2 μM; Sandoz, Basel, Switzerland) as an inhibitor of Pgp-mediated transport. For MRP, calcein-AM and probenecid (0.5 mM) served as substrate and inhibitor, respectively. P-glycoprotein and MRP functional assays were conducted as described by Feller et al.³⁶ Briefly, tumor cells (from the cell lines described already) were loaded with calcein-AM in medium A (growth medium without phenol red and bicarbonate buffer but with 20 mM Hepes) plus 10% fetal calf serum. After incubation, cells were washed in ice-cold medium A plus 10% fetal calf serum and incubated in drug-free medium plus 10% fetal calf serum, with and without the relevant modulator. The efflux was stopped by centrifuging the cells and adding ice-cold medium. Fluorescence was analyzed on a FACScan II. Results are expressed as the ratio of the two mean fluorescences (with and without modulator).

RESULTS

Immunohistochemical Analysis of Primary Choroidal Melanomas

The frozen sections of 12 primary choroidal melanomas were stained with two MAbs against different epitopes on each protein: Pgp (MAbs JSB-1 and C219), MRP (MAbs MRPM6 and MRPr1), and LRP (MAbs LRP-56 and LMR-5). JSB-1 showed slightly higher staining intensities, with higher background staining than C219 in each specimen; otherwise, no discrepancies occurred. Results of semiquantitative scoring (Table 1) are average scores of two antibodies per protein. P-glycoprotein was expressed weakly on the membranes of 4 tumors, and only 1 of 12 lesions showed significant staining. In two tumors, blood vessels stained positively with C219 and JSB-1.

Expression of MRP on tumors was higher: positive

or strongly positive staining was observed on 7 of 12 tumors, and only 1 tumor showed no immunoreactivity. Staining was predominantly cytoplasmic, but membrane staining was also observed in strongly MRP-positive tumors (Fig. 1). Immunoreactivity of LRP was the strongest of all MDR-associated proteins and occurred on all lesions, with 9 of 12 tumors scoring positive or strongly positive. No correlations were found between staining results and cell type or tumor size. Most tumors showed considerable heterogeneity of protein expression. These tumors were mostly rated "weak staining" or "positive staining" in Table 1. Lesions rated "strongly positive staining" showed more uniform staining. No relation could be determined with necrotic regions, vascularization, or pigmentation.

Detection of MDR-Associated Proteins on Cell Lines in Cytospins

The presence of the MDR-associated proteins was determined in five uveal melanoma cell lines. Staining was more homogeneous in cell lines than in tumor sections. Results of immunocytochemical analysis performed on cell lines are shown in Table 2 as average scores of staining results of two antibodies per cell line. On cytospin preparations, antibodies (4E3 and JSB-1) to Pgp showed little staining; Pgp was weakly detectable in three of five cell lines (92-1, OCM-1, OMM-1). MRP staining was observed on all cell lines, with a higher reactivity on EOM-3 than on the other cell lines. As in immunohistochemical analysis of the solid tumors, LRP expression was high: four cell lines showed high immunoreactivity, and only one cell line (Mel 202) showed weak reactivity. Staining of cytospins with LRP-56 showed a cytoplasmic granular pattern (Fig. 2), in accordance with reports of the cytoplasmic distribution of vaults.^{21,22}

Detection of MDR-Associated Proteins on Cell Lines in Flow Cytometric Analysis

Results of flow cytometric analyses are shown in Table 2. Monoclonal antibody MRK-16 was used to detect

FIGURE 1. Immunohistochemical detection of multidrug resistance-associated proteins in frozen sections of primary choroidal melanomas. Positive antigens are stained red. Cytoplasmic staining of lung resistance protein (LRP, *top left*) and multidrug resistance-associated protein (MRP, *top right*). There was no immunoreactivity to P-glycoprotein (Pgp) in the same specimen (*bottom left*). Negative (isotype-matched antibody) control is shown at bottom right. (Magnification, ×200.)

FIGURE 2. Detection of multidrug resistance-associated proteins on cell line EOM-3 in cytospin preparations. Positive antigens are stained red. Strong immunoreactivity of lung resistance protein (LRP) with a granular cytoplasmic pattern was observed (*top left*). Staining of multidrug resistance-associated protein (MRP) was predominantly cytoplasmic, with some staining of cell membranes (*top right*). No immunoreactivity of antibodies to P-glycoprotein was observed in EOM-3 (*bottom left*). Negative (isotype-matched antibody) control is shown at bottom right. (Magnification, ×400.)

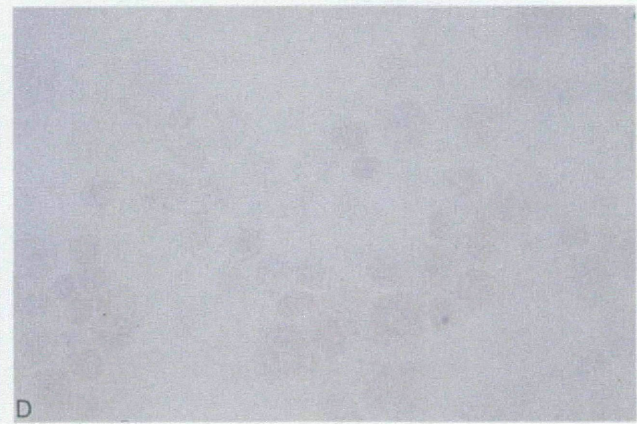
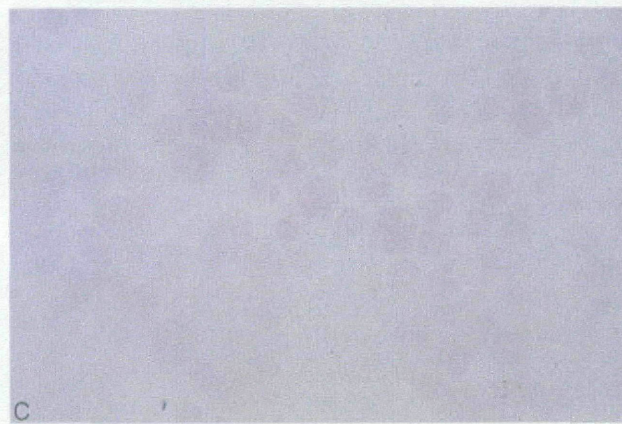
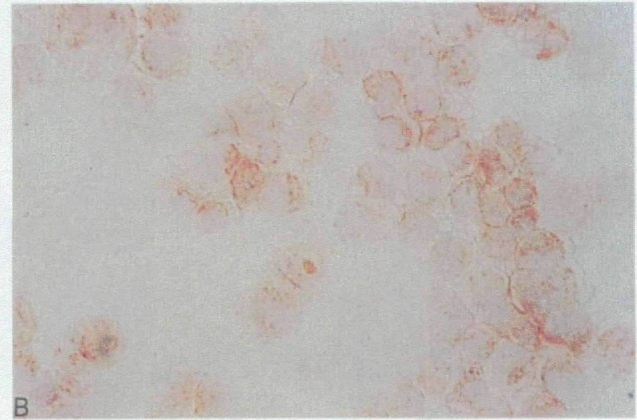
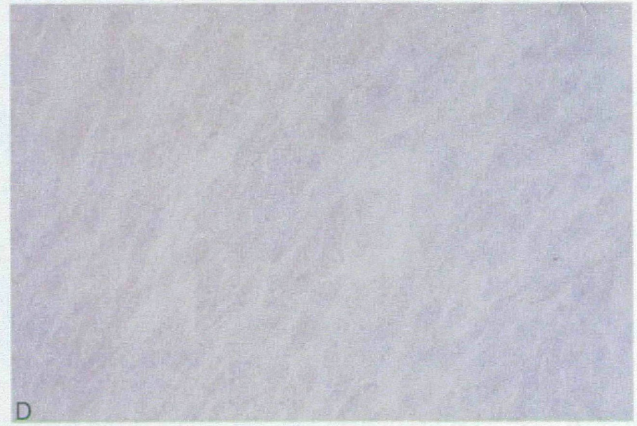
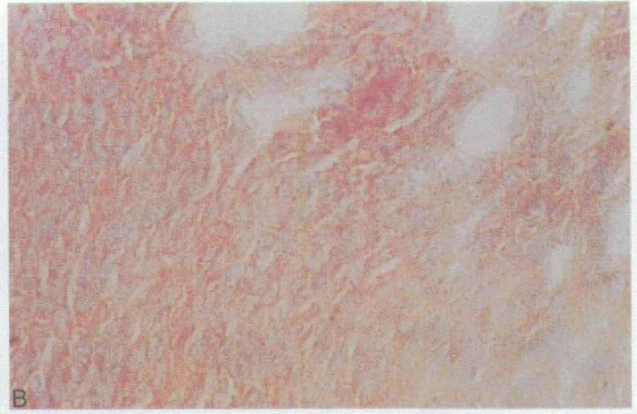
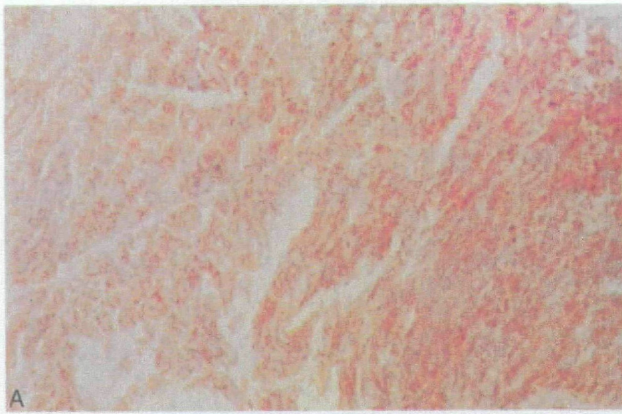


TABLE 2. Cell Lines: Results of Immunocytochemical and Flow Cytometric Measurements

Cell Line	P-Glycoprotein		MRP		LRP	
	Cyto-spin	Flow Cytometry	Cyto-spin	Flow Cytometry	Cyto-spin	Flow Cytometry
92-1	⊖	1.17	⊖	1.42	●	2.05
MEL202	○	1.22	⊖	1.79	⊖	1.75
EOM-3	○	1.14	●	1.60	●●	15.90
OCM-1	⊖	2.52	⊖	1.00	●●	4.80
OMM-1	⊖	2.54	⊖	1.32	⊖	2.20
Sensitivity	○	1.00*	⊖	2.90‡	○	3.75‡
Resistance	●●	13.30†	●●	15.3§	●	7.95§

Staining intensities of tumor cells in cytospin samples were scored according to the following scale: ○ = no staining; ⊖ = weak staining; ● = positive staining; ●● = strongly positive staining. Flow cytometry = ratios of mean fluorescences of specifically stained cells and negative controls. For interpretation of the results, ratios of known sensitive (limited expression of multidrug resistant [MDR]-associated proteins) and drug-resistant cell lines (high MDR-associated protein expression) are shown.

* Cell line KB3-1, sensitive to doxyrubicin and limited expression of P-glycoprotein.

† Cell line KB8-5 overexpresses P-glycoprotein and is twofold resistant to doxyrubicin.

‡ Cell line GLC4 has limited expression of MRP and lung resistance protein (LRP).

§ Cell line GLC4/ADR overexpresses MRP and LRP and is strongly drug-resistant.

the presence of Pgp on cell lines. Low levels of Pgp on cell lines OCM-1 and OMM-1 were observed, but the other cell lines showed no significant staining. MRP could not be detected on cell line OCM-1, and only weak immunoreactivity was demonstrated by flow cytometry in the other cell lines. In contrast, high levels of LRP were demonstrated on flow cytometric analyses. In cell line EOM-3, fluorescence ratios of LRP markers were twice as high as in the drug-resistant cell line GLC4/ADR, which was used as a positive control. Flow cytometry scatterplots showed variation in the size of tumor cells.

Detection of MDR-Associated Proteins on Cell Lines in Functional Assay

Some specific inhibition of efflux of rhodamine 123 and calcium-AM in the presence of PSC833 was detected in the functional assay of OCM-1, demonstrating a measurable transporter function of Pgp. No significant function of MRP could be detected in dye efflux measurements. Results are shown in Table 3.

DISCUSSION

Metastatic uveal melanoma shows a notably poor response to chemotherapy,² and the mechanisms conferring drug resistance to tumor cells in this disease are unknown. The discovery of MDR-associated transport proteins in other tumor cells has brought predictors of patient survival and has led to new possibilities for patient stratification, choice of drugs, and the addition of drug transport modulators to therapeutic protocols.¹⁰⁻¹⁴ These exciting studies demonstrate the clinical relevance of determining the specific drug-resistance phenotype of each different tumor. Expression of Pgp on choroidal melanomas was previously

described in a minority of tumors, in accordance with our results.³⁷ We report here for the first time on the expression of MRP and LRP on primary choroidal melanomas and cell lines. Our results show that the phenotype of uveal melanoma varies with regard to the expression of MDR-related proteins in type and level of expression. In general, LRP was very strongly expressed, Pgp and MRP weakly. Lung resistance protein was easily detected by all methods used; immunoreactivity of Pgp and MRP was strongest in immunocytochemical and immunohistochemical analyses.

Results obtained from in vitro cultured cell lines must be viewed critically because the phenotype of the tumor cells can change with the increasing number of passages. Therefore, results from sections of primary melanomas are probably more meaningful. In this study, the protein expression found in cell lines was comparable to that found in tumor sections.

The detection of these proteins in functional assays was not so evident; this could be expected because this technique is less sensitive than immunocytochemical analyses.^{36,38} In a functional assay, some specific inhibition of efflux of rhodamine 123 and calcium-AM by adding PSC833 was demonstrated in OCM-1, showing that on some uveal melanomas, Pgp has a measurable function as an adenosine triphosphate-driven pump. Compared with cutaneous melanomas, uveal melanomas appear to express slightly higher levels of Pgp.³⁹ Because in clinical studies of other tumors low levels of Pgp expression have demonstrated a significant influence on drug resistance and survival, this finding may be of clinical interest.¹⁴ For confirmation of such low levels of Pgp expression, however, determination of messenger RNA in tumor cells using the reverse transcription-polymerase chain reaction may be warranted.³⁸

TABLE 3. Cell Lines: Results of Functional Assays for P-Glycoprotein and MRP

Cell Line	P-Glycoprotein		MRP (Ca-AM)
	Rho	Ca-AM	
92-1	1.12	1.11	1.14
MEL202	1.01	1.01	1.12
EOM-3	1.01	1.09	1.04
OCM-1	1.51	1.27	1.06
OMM-1	1.13	1.15	1.18

Functional assay of P-glycoprotein: results (ratios of mean fluorescence) of two functional assays are given with rhodamine (Rho) and Calcein (Ca-AM) as substrates with and without PSC833 as inhibitor of pump function. Functional assay of MRP: results of Ca-AM measurements inhibited by probenidicid.

The present finding of high levels of expression of LRP in uveal melanoma (cell lines) suggests a role for LRP in the poor response of uveal melanoma to chemotherapy. LRP has been identified as the major human vault protein. Vaults are vesicle-associated, 13-MDa ribonucleoprotein particles composed largely of the (104-kDa) major vault protein, in addition to three (55-, 195-, and 210-kDa) minor vault proteins and vault RNA. These cellular organelles, discovered only 10 years ago, were found to be broadly distributed and highly conserved among diverse eukaryotic cells. Vault particles are thought to mediate transmembrane transport of various substrates, possibly in a fashion similar to that of proteosomes.^{21,22} Thus, vault organelles may mediate drug resistance by redistributing drugs away from the nucleus and extruding the drugs from the cell through exocytotic vesicles.

In this study, the intrinsic expression of MDR-related proteins varied greatly among primary uveal melanomas and also between different cell lines. This finding suggests a variation in drug resistance in these tumors and may in part explain incidental remissions by drug therapy in some patients. Patients may be stratified according to the presence or absence of MDR-related proteins on tumor cells. For clinical purposes, however, several issues must be resolved. The levels of expression of MDR-related proteins are strongly stimulated by exposure to cytotoxic drugs. This acquired resistance could be a significant factor in the clinical resistance of metastatic choroidal melanoma. Further, the phenotype of metastases may differ from that of the primary choroidal tumors described here.

This study indicates that different MDR-related proteins are intrinsically present on most primary choroidal melanomas, in accordance with known clinical resistance to cytostatic agents. However, our knowledge of drug resistance in metastatic choroidal melanoma is still limited, and additional studies are war-

ranted for better understanding of the high resistance of this disease to chemotherapy.

Acknowledgements

The authors thank H. J. Broxterman and P. van der Valk for their assistance and advice.

Key Words

cell lines, immunohistochemistry, melanoma, multidrug resistance, uvea

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