Multidrug resistance in ocular melanoma

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Abstract

Aims/background—Metastatic disease in patients with ocular melanoma is resistant to chemotherapy. One of the main mechanisms of modulating multidrug resistance is the expression of the multidrug resistance gene 1 (MDR1) product (p-glycoprotein) by tumour cells. The purpose of this study was to evaluate the frequency of expression of the MDR1 gene in ocular melanoma whose primary treatment was surgical excision or enucleation.

Methods—Twelve recent ocular melanomas were received fresh, snap frozen and cryostat sections of tumour were analysed for expression of MDR1 by immunohistochemistry using a well characterised monoclonal antibody to MDR1. Tumour explants were established in short term tissue culture from four tumours and cell blocks were examined by immunohistochemistry.

Results—MDR1 expression was present in five of 12 ocular melanomas. Upregulation of protein expression was found in four cell lines established in short term culture from tumour explants. A recurrent tumour, initially treated by local excision and radioactive plaque, showed overexpression of MDR1 mRNA.

Conclusions—These results suggest that significant levels of MDR1 may be intrinsically present in ocular melanomas before exposure to drugs involved in multidrug resistance, and indicate the possible importance of MDR1 in modulating chemoresistance in ocular melanoma. Chemosensitisation may be of potential value in planning adjuvant chemotherapy for patients with metastatic disease.

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The clinical behaviour of uveal melanoma is unpredictable.[1] Some tumours have a relatively benign behaviour while others are followed by early metastatic death.[1] Even a prolonged tumour-free interval does not rule out development of late metastases.[2] Once metastatic disease has developed, survival is measured in months. The median survival time after clinical detection of metastases is 9 months.[3] All currently used treatments are ineffective in prolonging survival,[4–6] which is at least partly the result of tumour resistance to chemotherapeutic agents. The exact mechanism of this resistance is unknown.

Multidrug resistance may be mediated by several mechanisms.[7] One of the best known is by expression of the multidrug resistance gene (MDR1) which encodes the drug efflux pump known as p-glycoprotein.[7 8] The purpose of this study was to determine (1) if MDR1 is expressed in primary ocular melanomas, (2) if expression can be inducible by in vitro culture methods, and (3) its relation to pathological variables. The methods used were immunohistochemistry of cryostat tumour sections, with a monoclonal antibody, and in one case mRNA expression was studied using reverse transcriptase and the polymerase chain reaction (RT-PCR).

Materials and methods

Since 1994 all ocular melanomas resected at the Royal Victoria Eye and Ear Hospital, Dublin, were immediately transported on ice to the laboratory, where the tumours were examined in a laminar flow hood. Part of the tumour was taken for short term tissue culture, and part was snap frozen in OCT and stored at −80°C until frozen sections were cut.

PRIMARY CELL CULTURE

Tumour samples were minced and digested for 4 hours in 1% collagenase. Cells were collected by centrifugation, grown in Ham's F-10 supplemented with 17% fetal calf serum, 3% horse serum, 2 mM glutamine, 100 U/ml penicillin-streptomycin, 2.5 µg/ml amphotericin B, 10 ng/ml epidermal growth factor and 10^{-8} M cholera toxin. The cells were passaged one in two at confluency. After a maximum of six subcultures, pelleted cells were embedded in 2% agar and processed through paraffin for immunocytochemical analysis.

IMMUNOHISTOCHEMICAL METHODS

Cryostat sections of 5 µm were cut, dried overnight at room temperature, fixed in acetone (10 minutes at room temperature), and stored at −80°C until use. Sections were allowed to thaw for 2 hours at room temperature before immunohistochemical staining. The primary antibody used was the monoclonal antibody BRI MAB MDR1 clone 6/1C (supplied by E Moran and A Larkin, National Cell and Tissue Culture Centre/BioResearch Ireland, Dublin City University, Dublin 9, Ireland). The dilution used was 1:100 in 1% BSA in phosphate buffered saline at pH 7.4. The antibody has been shown to be specific for the MDR1 gene product (E Moran, personal com-
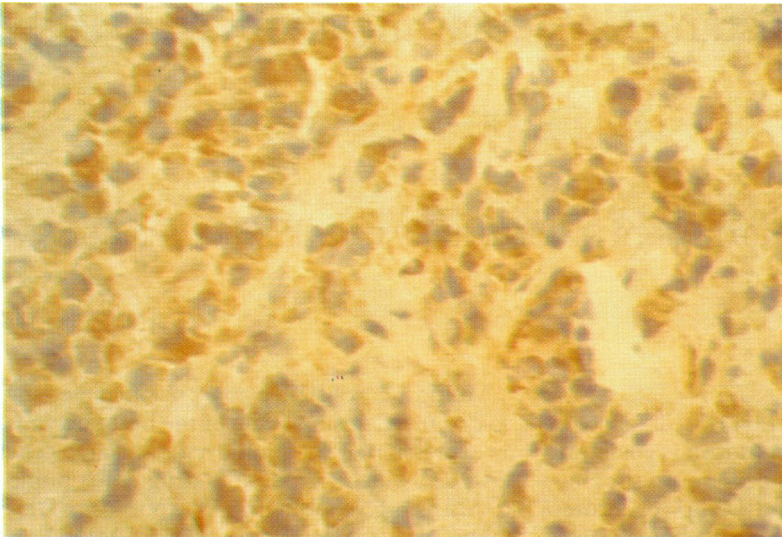
Figure 1   Cytoplasmic immunoreactivity for MDR1 is seen in a majority of tumour cells from case 2 (original magnification × 40).

munication). Incubation with the primary antibody was for 18 hours at 4°C. A standard avidin-biotin-peroxidase technique was used[9] and a Vectastain Elite murine ABC kit (Vector Labs, Burlingame, CA, USA). All incubations were carried out as per kit instructions and 3'3' diaminobenzidine tetrachloride was used as a chromogen. This gives a brown reaction product. In order to confirm that the brown immunoreaction product could not be mistaken for intracytoplasmic melanin within the tumour cells, all reactions were repeated using an alkaline phosphatase method (APAAP) with an APAAP kit from Vector laboratories and fast red salt (Vector red) as the chromogen. All sections were counterstained using Harris's haematoxylin, dehydrated, cleared, and mounted. The negative control used was omission of the primary antibody. The positive control was a breast cancer sample shown to express MDR1 mRNA by reverse transcriptase PCR. The test slides were compared with negative and positive control sections and were scored as positive when an unequivocal positive reaction product was seen within tumour cells on the test slides. There was no staining of any of the negative control slides. Semiquantitative evaluation was performed by counting all cells and all positive cells in 10 microscopic fields (× 400). The percentage of positive cells was calculated and graded 0 when no tumour cells

stained; 1 when 1–10% of cells were positive; 2 when 11–50% were positive; and 3 when 51–100% of cells were positive.

RT-PCR

A single pair of pre- and post-radiation treated tumour samples was available only in formalin fixed paraffin embedded tissue blocks. Sections of 50 μm were cut, total cellular RNA was extracted, quantified, and cDNA was synthesised by reverse transcription as previously described.[10 11] As soon as the cDNA copy was generated using mRNA as a template, the polymerase chain reaction was set up so that all reactions produced amplification of a control gene (β actin) and the target gene (MDR1). β actin is constitutively expressed in all cells. The concentration of control primers was 125 μg/μl and of target gene was 250 μg/μl. The reactions were carried out as follows: an initial denaturing step of 94°C for 1.5 minutes. Thirty cycles of PCR amplification were performed: a denaturing step at 94°C for 1.5 minutes, an annealing step at 4°C for 1 minute, and an extending step at 72°C for 3 minutes. The amplified fragments were identified by gel electrophoresis and ethidium bromide staining.

Results

CLINICAL DETAILS

Twelve tumours were obtained fresh (cases 1–12) for this study. Fresh tissue was not available for case 13. The original presentation of this patient's tumour was in 1992 when she was treated by local resection and plaque radiotherapy for a 18 mm choroidal tumour with vortex vein invasion. The tumour recurred in a circumferential pattern in 1995, necessitating enucleation. Paraffin embedded and formalin fixed tumour tissue were available from both specimens for mRNA estimation using RT-PCR, instead of immunohistochemistry. Agar embedded cell blocks were prepared from successful in vitro cultures of cases 2, 4, 7, and 12 as detailed in Table 1.

The clinical details of all cases are summarised in Table 1. Eight of the patients were female and five were males. Their ages ranged from 50 to 84 years. Two tumours arose in the conjunctiva, two in the ciliary body, eight in the choroid, and one in the orbit. The tumours varied in size from 11 to 24 mm. Of the 10 intraocular tumours, five had invaded the sclera, one had extrascleral deposits, and two had invasion of the vortex vein at the time of enucleation or resection. None of the patients was suffering from detectable metastases.

IMMUNOHISTOCHEMICAL RESULTS

Five of 12 tumours showed immunoreactivity with cytoplasmic diffuse staining of tumour cells. One tumour was graded 1 and four were graded 3. Figure 1 illustrates the immunoreactive tumour in case 2. All of the four cultured tumours demonstrated immunoreactivity, while two of the four corresponding primary tumours were positive. Figure 2 illustrates immunoreactivity in cell blocks derived from a cultured primary tumour. Four of five tumours classified as epithelioid cell tumours were

Table 1 Clinical details of cases

Case No	Age/sex	Site	Cell type	Size (mm)	Scleral invasion	MDR1
(1)	50/F	Conj	E	16	NA	3
(2)	56/M	Chor	E	11	Y/vv	3
(3)	84/F	Chor	M	24	Y/ES	0
(4)	69/F	Chor	S	12	Y	0
(5)	70/F	CB	E	17	Y/vv	3
(6)	69/M	Chor	M	12	N	1
(7)	63/M	Orbit	E	21	NA/mets	3
(8)	75/M	Chor	S	17	N	0
(9)	57/F	Chor	S	11	N	0
(10)	51/M	Chor	S	18	N	0
(11)	77/F	Conj	E	10	NA	0
(12)	74/F	CB	M	20	Y	0
(13)*	53/F	CB/chor	M	17	Y	NA

NA = not applicable; N = no; *post-radiotherapy, original tumour 1992; CB = ciliary body; Y = yes; Chor = choroid; ES = extrascleral invasion; Conj = conjunctiva; mets = systemic metastases; vv = vortex vein invasion.
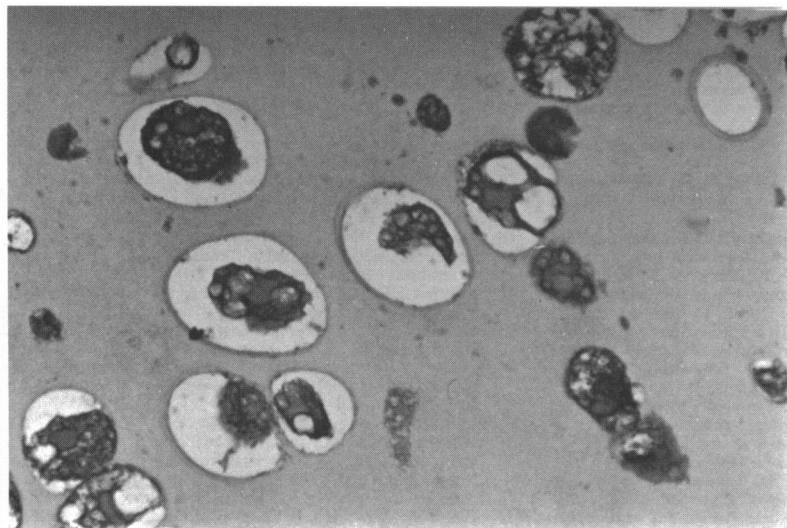
Figure 2   Expression of MDR1 is illustrated in cultured tumour cells prepared from case 11 (original magnification × 60).

immunoreactive for MDR1. One of two mixed tumours was immunoreactive and none of the purely spindle tumours were positive. Tumours with either vortex vein invasion or metastatic disease also showed immunoreactivity, but there was an absence of immunoreactivity in those three cases in which invasion was confined to the sclera.

RT–PCR
Evidence for expression of the MDR1 gene was detected in the post-treatment specimen, but not in the pretreatment sample. Amplification of a control gene, β actin, was found in both pre- and post-treatment samples (Fig 3).

Discussion
Multidrug resistance is the term used to describe the process whereby a tumour becomes resistant to a variety of drugs. One of the main mechanisms of drug resistance is by overexpression of the MDR1 gene which encodes a 170 kDa transmembrane glycoprotein known as p-glycoprotein.[7] This is a membrane efflux pump protein which keeps intracellular drug concentrations low by pumping drugs out of the cell. Other non-MDR1 related mechanisms include ribosomes, microtubules, DNA topoisomerase II,
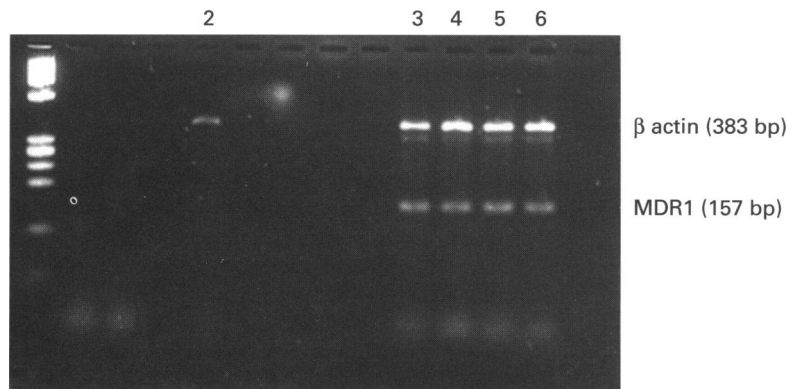

Figure 3   Ethidium bromide stained gel showing in lane 2 amplification of β actin (control gene) only in the pre-radiation tumour. Lanes 3, 4, 5, and 6 demonstrate amplification of both β actin and MDR1 in four separate aliquots of post-radiation tumour.

and detoxifying enzymes such as glutathione transferases.[7]

Expression of MDR1 has been found in a minority of primary skin melanomas and metastatic malignant melanomas by immunohistochemistry.[12] However, 45% of skin melanomas expressed MDR1 at low levels when sensitive techniques, such as amplification of messenger RNA by polymerase chain reaction, were used.[13]

In the present study we have found by immunohistochemistry that approximately 42% of melanomas of ocular origin expressed the MDR1 gene, indicating that a sizeable number of these tumours have intrinsic expression. A similar finding of intrinsic MDR1 expression was found in an immunohistochemical study of breast cancers.[14] Intrinsic expression may potentially be upregulated following chemotherapy. This is of clinical relevance, because drugs likely to induce MDR1 should be avoided if possible. The effects of MDR1 can be blocked by the use of competitive inhibitors such as the calcium channel blocker verapamil, calmodulin antagonists, or other inhibitors.[7] In the present study, increased expression of MDR1 was found following primary in vitro cell culture. The cell culture medium included the biological modifier cholera toxin, which may have contributed to upregulation of MDR1.

Although the numbers are small in this series, there was a trend towards MDR1 expression in tumours which have adverse prognostic factors: larger size and epithelioid cell type.[15-17] Some studies have shown that extrascleral extension per se is not an independent prognostic factor but is rather a marker of adverse cell type.[18] These findings may indicate that MDR1 expression is prognostically important. MDR1 has recently been shown to be correlated with clinical outcome in osteosarcoma[19] and to be of prognostic relevance in Ewings' sarcoma and neuroblastoma.[20 21]

In conclusion, MDR1 expression was detected by immunohistochemistry in 41.6% of ocular melanoma tissue specimens. This mechanism appears to have some importance in modulating drug resistance in ocular melanoma. Use of more sensitive techniques such as RT-PCR would probably identify a larger number of tumours expressing MDR1. Further studies with a larger series of cases and paired pre- and post-treatment biopsies are necessary to determine whether expression of MDR1 is of prognostic value and whether expression is increased following chemotherapy or radiation therapy. This has obvious clinical relevance in the choice of chemotherapeutic agents for metastatic disease and would justify inclusion of chemosensitising agents in the treatment of patients with metastatic disease.

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