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Immunohistochemical demonstration of metallothionein in eyes with choroidal melanomas

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PURPOSE. *The since immunohistochemically detectable metallothionein (MT) overexpression has been described in a variety of human tumours, including skin melanomas, in relation to different stages of tumour development and progression.*
MATERIALS AND METHODS. *We used a monoclonal antibody to investigate the distribution of MT in 18 formalin-fixed, paraffin-embedded, surgically enucleated eyes with choroidal melanomas, from 18 patients (8 male, 10 female; age range 30-83 years, mean 58.7). Clinico-pathological details and follow-up data (2-124 months, mean 36.1) were also available. MT immunoreactivity was recorded and the percentage of stained cells was graded for semiquantitative purposes. Correlations between immunohistochemical data and morphological characteristics of melanomas were investigated using non-parametric methods; survival analysis was done by the Kaplan-Meier method and the survival curves were compared by the Mantel-Cox log-rank test.*
RESULTS. *MT immunoexpression was found in 15/18 cases (83.3%) with staining scores from 1 to 3; MT staining varied in intensity and was mainly localized in the cytoplasm, although a combined nuclear/cytoplasmic reactive pattern was seen in neoplastic elements. No differences in MT immunostaining were seen in relation to age or sex, tumour size, histotype and amount of pigment; univariate analysis of survival data showed no prognostic significance regarding MT expression.*
CONCLUSIONS. *The immunohistochemical evidence of MT in neoplastic elements could be related to the production of this scavenging protein in the tumour for cell defense mechanisms against hydroxyl free radicals, and to act as a Zn donor, since Zn is required for the synthesis of DNA and DNA-repair enzymes. (Eur J Ophthalmol 2000; 10: 312-7)*

KEY WORDS. *Choroidal melanoma, Metallothionein, Immunohistochemistry*

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INTRODUCTION

Metallothionein (MT) is a low molecular-weight protein (6-7 kD) with a high content of cysteinyl residues and strong affinity for divalent heavy metal ions such as zinc, copper, cadmium, mercury, silver and platinum (1, 2). MT is believed to be involved in the storage of essential metals (3-5), binding large amounts of potentially toxic metal ions with a sequestration function (2, 5-7), and scavenging free radicals in tissues and cells (8). By immunohistochemistry, MT has been demonstrated in both the nucleus and the cytoplasm of cells in various organs of humans (9-11) and animals (2, 12-17).

In human neoplastic pathology (18), MT has been immunocytochemically detected in testicular embry-

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onal carcinomas (19), thyroid tumours (11), transitional cell carcinomas of the bladder (20), *in situ* and invasive breast carcinomas (5, 21), cervical intraepithelial and invasive squamous carcinomas (22), skin malignant melanomas (23), hepatocellular (24), pancreatic (25) and colorectal (26, 27) carcinomas. Although MT overexpression is mainly associated with the more malignant higher-grade tumours, besides with shorter survival and with local tumour recurrence (5, 18, 23, 25), the clinicopathological significance of MT has not been adequately assessed in other tumours (11, 18-20, 26, 27).

MT distribution in the eye has been systematically investigated only in rats (17), in which strong immunostaining was observed in the epithelium of the lens and cornea, in the retinal pigment cell layer and in glial cells of the optic nerve (17). PCR studies have shown a decrease in MT synthesis in early-onset macular degeneration in cynomolgus monkeys (28). On the light of these findings, we used a monoclonal anti-MT antibody reactive against a single and highly conserved epitope shared by the I and II isoforms, to analyse the immunohistochemical distribution pattern of MT in human choroidal melanoma, the most common intraocular neoplasm.

MATERIALS AND METHODS

Surgical samples of ocular globes taken from 18 patients with choroidal melanomas (8 male, 10 female; age range 30-83 years, mean age 58.7) were studied. The clinico-pathological data and characteristics of melanomas according to McLean et al (29, 30) are summarised in Table I. Follow-up data and causes of death were available from city registry offices.

All samples were fixed in 10% neutral formalin for 24-36 h at room temperature and embedded in paraffin at 56°C. From each tissue block, 3 µm thick sections were cut, mounted on silane-coated glass, dewaxed in xylene, rehydrated in graded ethanols, then immersed in 600 ml of 0.01 M sodium citrate buffer

TABLE I - CLINICO-PATHOLOGICAL DATA OF CHOROIDAL MELANOMAS

Case	Sex	Age	Histotype	Amount of pigment	Diameter (mm)	Thickness (mm)	Size	MT score
1	M	72	NS	Mo	8	11	L	1
2	F	70	NS	Mo	13	9	Me	2
3	F	61	S	Mo	11.5	6	Me	2
4	F	45	NS	A	5	1.5	S	0
5	M	30	S	A	14	7	Me	1
6	M	67	S	A	4	2	S	2
7	F	78	NS	Mo	5	8.5	Me	0
8	M	62	NS	Mo	14	11	L	1
9	F	50	NS	Mo	13	4	Me	2
10	M	49	NS	Mo	10	10.5	L	2
11	M	55	NS	R	11	8	Me	2
12	M	56	NS	Mo	10	11	L	3
13	F	44	NS	R	6	3.5	Me	2
14	F	83	NS	R	20	9	L	0
15	F	65	S	R	16	14	L	2
16	F	81	NS	R	22	2	L	2
17	M	30	S	Mo	15	9	Me	1
18	F	59	S	Mo	6.5	4	Me	3

MT= Metallothionein; M= Male; F= Female; NS= Non-Spindle cells; S= Spindle cells; A= Absent; Mo= Moderate; R= Rich; S= Small; Me= Medium; L= Large; MT Score: 0 (No staining); 1 (>0 to 5% stained cells); 2 (>5 to 25% stained cells); 3 (>25 to 50% stained cells)

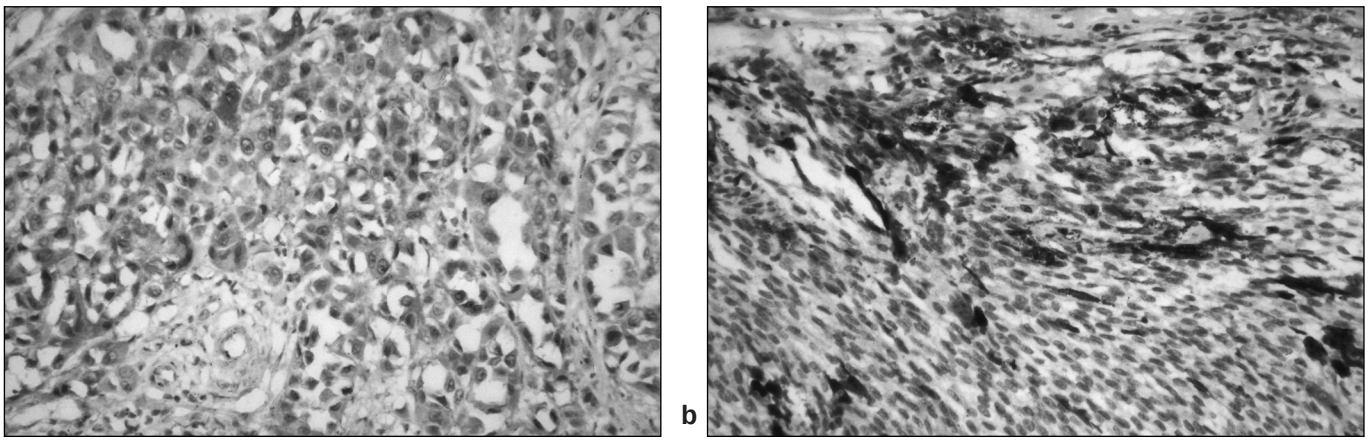

Fig. 1 - *Choroidal melanomas - Diffuse immunoreactivity was evident in amelanotic areas (**a**, 50x); scattered immunopositive elements were encountered in pigmented spindle-cell melanoma (**b**, 50x) (Mayer's hematoxylin counterstain).*

(pH 6.0) in an 850 W domestic microwave oven; sections were microwaved for 3 cycles of 5 minutes, allowed to cool for 20 minutes and soaked in phosphate buffered saline (pH 7.4) before immunostaining. For the immunoreaction a monoclonal mouse anti-MT reactive against a single and highly conserved epitope shared by the I and II isoforms was commercially obtained (Dako, Denmark, w.d. 1: 100) and applied overnight at 4°C. The tissue binding of the antibody was disclosed using a standard alkaline phosphatase anti-alkaline phosphatase technique employing new fuchsin as the substrate; finally, slides were slightly counterstained with Mayer's hematoxylin. To test the specificity of MT staining, the specific MT-E9 antiserum was omitted or replaced by either phosphate buffered saline or normal rabbit serum: negative results were obtained. Normal human kidney sections served as positive controls.

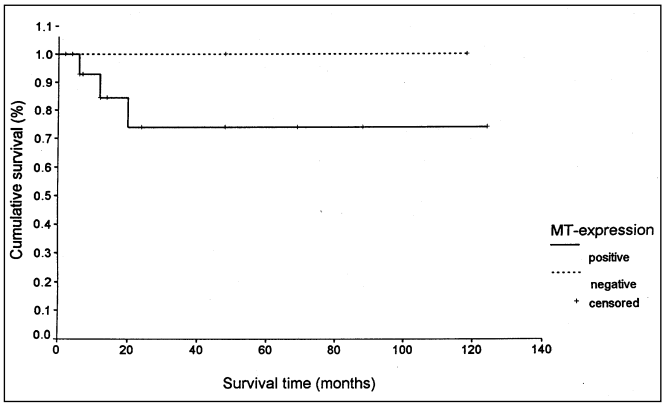

Fig. 2 - *Kaplan-Meier survival curves for MT positive and negative cases.*

Immunostained sections were examined by light microscopy using x20 and x40 objective lenses and x10 eyepiece. Immunostained sections were assessed on a consensus basis by two pathologists (G.T. and G.G.), using a double-headed microscope. Stained cells were

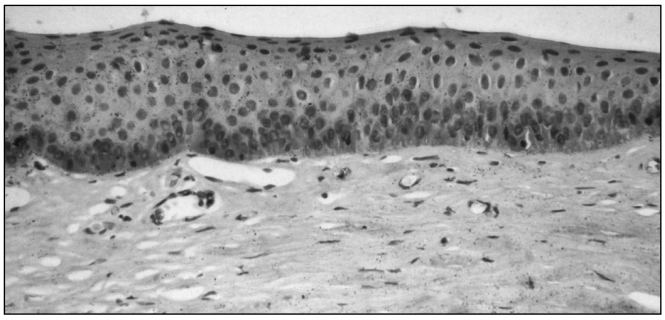

Fig. 3 - *In the corneal epithelium staining for MT was mainly in the basal layer (50x, Mayer's hematoxylin counterstain).*

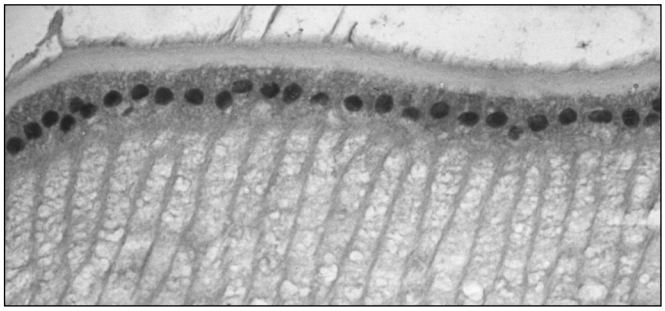

Fig. 4 - *Cytoplasmic immunostaining was evident in the epithelium of the lens (100x, Mayer's hematoxylin counterstain).*

graded for semiquantitative purposes as follows: 0 (no staining); 1 (>0 to 5%); 2 (>5 to 25%); 3 (>25 to 50%); 4 (>50%). Correlations between immunohistochemical data and morphological characteristics of melanomas and between individual parameters and death due to melanoma were investigated using non parametric methods (chi-square or Fisher's exact test, Mann-Whitney *U*-test, Kruskal-Wallis *H*-test). Survival analysis was done by the Kaplan-Meier method, grouping the patients in two different ways: the first comparing MT-negative cases with MT-positives, the second comparing cases with 0 and 1 score and cases with 2 and 3 score. The Mantel-Cox log-rank test was used to compare survival curves. A p value less than 0.05 was considered statistically significant.

RESULTS

MT immunoexpression was found in 15/18 cases (83.3%) with staining scores from 1 to 3 (Fig. 1 a, b); 9/15 melanomas had from 5 to 25% stained elements (Fig. 1a). None of cases gave a MT score of 4. The intensity of MT staining was variable, but it was always mainly in the cytoplasm, although a combined nuclear/cytoplasmic reactive pattern was seen in neoplastic elements. Frequently immunoreactive neoplastic cells were in direct contact with negative ones (Fig. 1b).

Non-parametric statistical methods showed no differences in MT immunostaining in relation to age, sex, or pathological parameters such as tumour size, histotype (spindle or non-spindle) and amount of pigment.

Follow-up ranged from 2 to 124 months (mean 36.1). Three patients died of disease with metastasis (1 liver, 1 liver and brain, 1 liver and adrenal glands), while 15 were well and alive. Chi-square or Fisher's exact test showed no correlations between morphological or individual parameters and death due to melanoma. No significant differences in MT expression were seen in the comparison of patients still alive or deceased. Univariate analysis of survival data, considering MT-positive and negative cases or grouping patients with different MT scores, did not achieve prognostic significance (Fig. 2).

In all eyes, MT immunoreactivity was evident in the epithelium of the cornea, with a stronger staining mainly in the epithelial basal cell layer (Fig. 3). In addition, the epithelium of the lens was greatly reactive with MT antiserum (Fig. 4), while the capsule and equatorial regions were unstained. In the retina MT expression was appreciable in the nerve fiber and inner plexiform layers, with more pronounced staining in the pigmented epithelium.

DISCUSSION

Malignant melanoma develops more frequently in the uveal tract of the eye than in any other site of the body except the skin; it is the most common intraocular neoplasm and its incidence is one-eighth that of cutaneous melanoma (29, 31). In this latter, MT is positive in either the nucleus or cytoplasm of neoplastic cells and its presence has been significantly associated with progressive disease and the vertical thickness of melanomas (23). In choroidal melanomas, we encountered combined nuclear-cytoplasmic MT immunoexpression in 83.3% of cases, a percentage similar to that observed in cutaneous melanomas with thickness >1.5 mm (23); the MT staining score ranged from 1 to 3 with variable degrees of staining intensity. This immunohistochemical evidence of MT in neoplastic elements may be related to the production of this scavenging protein in the tumour itself to set up cell defense mechanisms against hydroxyl free radicals (8), and also for the homeostatic control of Zn and Cu (32). Exchanges between Zn-MT and the apoform of Zn-enzymes MT have been reported *in vitro* (33), suggesting MT is involved in metal-transfer reactions *in vivo*. Therefore, the immunohistochemical expression of MT in neoplastic elements might be related to a role of MT as a Zn donor to these apoenzymes, since Zn is required for synthesis of DNA and DNA-repair enzymes (8). The absence of MT immunoreactivity in three of the 18 ocular melanomas studied should be attributable to the production of other MT isoforms during carcinogenesis as a consequence of different gene expression. In humans ten functional MT isoforms and seven non-functional ones have been reported as results of a family of genes (34). The commercially available antiserum we used reacts only against a conserved N-terminal epitope shared by MT-I and MT-II isoforms and is therefore unable to distinguish between the MT-I and MT-II isoforms or metal-bound and metal free forms of MT, or to detect

other MT isoforms, as suggested elsewhere (18).

Many factors influence the survival of patients with choroidal melanomas and may predict the appearance of distant metastases (29, 35); cell type, largest tumour dimension, scleral extension and mitotic activity, in combination, have been considered the best prognostic indicators (36, 37). However, the relationship between MT expression and prognosis in human tumours is not fully understood and conflicting explanations have been offered (5, 21-27). A direct correlation between MT expression and poor clinical outcome was found in invasive breast ductal carcinomas (5) and malignant melanomas of the skin (23); by contrast, no correlation was established with tumour differentiation or tumour aggressiveness in MT-positive thyroid (11) and testicular embryonal carcinomas (19). Finally, a favourable clinical outcome of MT-positive colonic carcinoma has been suggested (26), even though we found MT immunoreactivity in stage I and II colorectal carcinomas and in advanced ones, as well as in metastases of these latter, thus excluding a relationship between MT staining and a better prognosis (27).

In the present study, the immunohistochemical evidence of MT in choroidal melanomas did not indicate any difference between small, medium and large melanomas, between intensely or less pigmented lesions, or between spindle and non-spindle histotypes; moreover, no correlation was found between MT immunostaining and age or sex of patients. Survival curves for patients with positive or negative MT expression were not significantly different even when immunohistochemical data were considered in groups with low or high MT content. Therefore, the use of MT immunopositivity as a prognostic parameter in choroidal melanomas must still be viewed with caution, requiring more extensive investigation.

An additional interesting finding was the MT positivity in unaffected compartments of the eyes we studied. Strong immunoreactivity was observed in the epithelium of the cornea and lens and in the retinal pigment cell layer. Since dividing cells migrate from the basal layer to the corneal surface for turnover (38), and the lens epithelium shows a high proliferation capacity under normal and pathological circumstances (38), the presence of MT in these cells may indicate its association with cell proliferation and maturation. However, MT may also scavenge the free oxygen radicals produced in the cornea and lens by ultraviolet and X-ray radiations. A similar detoxifying role is possible for MT in the pigment cell layer, to prevent the influx of toxic substances to the retina.

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