TUMOR PROGRESSION IN UVEAL MELANOMA

(Tumor Progressie in het Melanoom van de Uvea)

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TUMOR PROGRESSION IN UVEAL MELANOMA

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ter herinnering aan mijn vader

aan mijn moeder

aan Gerard, Wouter en Rolf

CONTENTS

Abbreviations

AgNOR	Silver staining of the nucleolar organizer region
BDUMH	bilateral diffuse uveal melanocytic hyperplasia
CAM	cellular adhesion molecule
Cdk	cyclin-dependent kinase
CEA	carcino embryonic antigen
CK	cytokeratin
CV	coefficient of variation
EGF	epidermal growth factor
ELAM	endothelial leucocyte adhesion molecule
FACS	fluorescence activated cell sorter
FAMM	familial atypical multiple mole melanoma
FCM	flow cytometry
Gy	gray
HLA	histocomptability antigens
HMW	high molecular weight
HPF	high power field
ICAM	intercellular adhesion molecule
Ig	immunoglobulin
IL	interleukin
Ir	Iridium
kD	kilodalton
LFA	leucocyte function-associated antigen
LTD	largest tumor diameter
mRNA	messenger RNA
MAA	melanoma-associated antigens
MAb	monoclonal antibody
MLN	mean of the largest nucleoli
NCAM	neural cell adhesion molecule
NOR	nucleolar organizer region
PA	plasminogen activator
PAI	plasminogen activator inhibitor
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
SDNA	standard deviation of the nucleolar area
SPSS	statistical package for the social sciences
Td	tumor doubling time
TGF	transforming growth factor
TIL	tumor-infiltrating lymphocytes
TNF	tumor necrosis factor
t-PA	tissue-type plasminogen activator
u-PA	urokinase-type plasminogen activator
u-PAR	urokinase plasminogen activator receptor
UV	ultraviolet
VCAM	vascular cell adhesion molecule
VLA	very late activation antigen

CHAPTER 1

1.1 General Introduction

Ophthalmic melanomas can be divided in extra-ocular (conjunctiva, caruncle) and intra-ocular uveal melanomas (iris, ciliary body and choroid). Uveal melanomas account for 95% of ocular melanomas, while only 5% are conjunctival in origin. The extra-ocular and intra-ocular melanomas differ in biological behavior. Melanocytes originate from the neural crest and are normally present in the uveal stroma, similar to dermal melanocytes. Intra-epithelial precursor lesions of invasive melanoma occur in the conjunctiva and the caruncle but within the eye an intra-epithelial (retinal pigment epithelium) precursor lesion has not been demonstrated. Therefore a radial and vertical growth phase, as is recognized in cutaneous and conjunctival melanomas, is not evident in intra-ocular melanoma. Because uveal melanomas are not easily accessible for incisional biopsy (without disruption of vision), only two lesions of melanocytic origin are defined clinically: nevus and melanoma.[1] Progression in melanoma is clinically associated with tumor size. Most malignant melanomas of the choroid can be diagnosed by ophthalmoscopy and ultrasonography, and evidence of growth is best established by serial photography of the fundus. The uvea consists of highly vascularized tissue. There are no demonstrated lymphatics within the uveal tract, or in the posterior orbit. This explains the difference in biological behavior of ophthalmic melanomas: conjunctival melanomas spread first to regional lymph nodes whereas choroidal and ciliary body melanomas metastasize hematogenously and preferentially first to the liver. Strikingly, another intra-ocular tumor, the retinoblastoma usually does not metastasize until after it has invaded the orbit. From the orbit it gains access to lymphatic vessels in the anterior orbit. Unlike uveal melanoma, the initial metastases from retinoblastoma are often to regional lymph nodes. Uveal melanoma metastasize relatively late: the 5, 10 and 15-year survival rates based on tumor-related deaths vary from 65%, 52% and 46%, respectively;[2,3] to 72%, 59% and 53%, respectively, in recent series.[4,5,6] The estimated 5-year-survival rate of cutaneous melanoma varies between 70-80%.[7,8] Once the diagnosis of hepatic metastasis from uveal melanoma has clinically been made, the median survival is extremely poor: between two [9,10] and seven months.[11] The median survival time in patients in whom the liver was either not involved at all, or not among the first sites of dissemination is 19 months.[11]

The question remains how a (histologically) malignant melanoma acquires metastatic capacities and seeds malignant cells preferentially to some organs. Organ-specific

colonization by malignant cells often follows very specific interactions between the cancer cell and the target organ, either in terms of specific cellular adhesion or growth promotion.[12,13,14] A metastasizing cell must break loose from its parent tumor, invade the matrix between cells and penetrate the wall of a blood vessel. It must pass through the blood stream, escape the immune system and emerge from the bloodstream at a favorable spot, lodge and induce growth of new blood vessel growth. However, the molecular differences between metastasizing and non-metastasizing uveal melanoma cells are not well known. The products of oncogenes also play a role in the metastatic competence of a tumor. Proto-oncogenes are normal genes that, when activated by mutation or translocation, are called oncogenes which contribute to tumorigenesis. It is still not known to what extent or in what way oncogenes control the acquisition of cancerous properties or if they control the manifestation of metastasis.[15]

1.2 Aim of the Thesis

This thesis deals with melanoma of the posterior uveal tract (choroid and ciliary body); in particular with progression of melanomas, defined as the acquisition of irreversible changes in the tumor, gaining metastatic potential. For patients with uveal melanoma there is no effective therapy once metastases have developed. Survival time for treated, but possibly uncured patients is related to the rapidity of the metastatic process. In order to lower melanoma-related mortality, it is essential to prevent or eradicate metastatic disease. This calls for early detection and for the development of reliable prognostic factors. When an effective systemic treatment for metastatic uveal melanoma would be available, early administration as an adjuvant to primary treatment may provide the best strategy for control of systemic spread. It is therefore necessary to increase our knowledge of the mechanisms underlying metastasis, and the identification of reliable progression parameters as prognostic markers in primary uveal melanoma.

Our studies, described in this thesis, have focused on the clinicopathological characteristics and the possible role of the products of oncogenes in progression of uveal melanoma. In chapter 2 current knowledge on the epidemiology, histology, conventional prognostic factors, classification and grading, and survival of human uveal melanoma is summarized. The uncommon, relatively benign iris melanoma is shortly described in 2.2. In contrast, posterior (choroidal and ciliary body) melanomas have a relatively poor prognosis. The clinical symptomatology, growth patterns and indications for aspiration biopsies of these

tumors are described in 2.3. In chapter 3 the relevant literature on research on prognostic factors in uveal melanoma is reviewed. In chapter 4 a rare precursor of uveal melanoma, i.e. atypical melanocytic hyperplasia developing into low grade malignant melanoma is described. To investigate the possible UV mediated N-*ras* oncogene activation, uveal melanomas were analyzed for N-*ras* point mutations (chapter 5). Pre-enucleation radiotherapy has been introduced to lower the risk of hematogenous seeding during the enucleation procedure.[16] We investigated the effect of pre-enucleation radiotherapy on proliferation (chapter 6) and DNA-ploidy (chapter 7). In the search for prognostic factors we investigated the prognostic value of DNA-ploidy (chapter 7), proliferation parameters and tumorsuppressor gene expression (chapter 8). Furthermore we studied the role of the plasminogen activator system (chapter 9) and the expression of neural cell adhesion molecules (NCAM) in the acquisition of malignant properties of uveal melanoma (chapter 10).

References: page 37

CHAPTER 2: Malignant Melanoma of the Uveal Tract

2.1 Epidemiology

2.1.1 Incidence and geographic factors

The epidemiologic aspects of uveal melanoma have been extensively reviewed by Egan et al.[17] Melanoma of the uvea is the most common primary intraocular malignancy in adults.[18] The estimated incidence is six cases per one million subjects per year in the western population.[2,19,20,21] The incidence of uveal melanoma in whites is eight times that in blacks and threefold greater than that in certain Asian groups.[22] In the Caucasian population individuals with light irides have three times the risk of developing uveal melanoma compared to persons with brown eyes.[23] Early life exposures to sunlight have been found to be especially important in the development of intra-ocular melanoma.[24] Recent epidemiological studies have reported an elevated risk for Northern European ancestry, light skin color, the presence of 10 or more cutaneous nevi, use of sunlamps and intense sun exposure.[25] Holly found an increased risk of developing uveal melanoma for the apparent effects of UV exposure (severe eye burn, snow blindness), and for host factors, like eye color and a propensity to burn rather than tan.[26] These findings implicate sunlight as an environmental risk for this disease. It is believed that the choroid and ciliary body are protected from UV exposure and also from a large portion of the more energetic wavelengths of the visible spectrum by the overlying retina and retinal pigment epithelium.[27] We must, however, conclude that although there is ample epidemiological evidence for a role of UV radiation as a risk factor in developing uveal melanoma, it is unlikely that UV radiation is able to reach the choroid. The exposure to sunlight would also not explain the pathogenesis of melanomas arising in the ciliary body that is not in the direct path of light entering through the pupil, but may be reached by scattered light. For iris melanomas the role of sunlight seems more convincing: iris melanomas tend to occur in the inferior sector of the iris.[28] The upper eyelid shields the superior half of the iris from sunlight. The tendency for this tumor to appear in blue-eyed individuals is also supportive of the role of actinic damage in these tumors. However the incidence of iris melanomas is much smaller compared to those arising in the ciliary body or choroid. Another argument against the direct role of UV radiation in uveal melanoma might be that incidence and mortality rates for uveal melanoma are changing very little in Europe, North America, Japan and Australia.[29] This finding is in contrast to the rapid increase of the incidence of cutaneous melanoma.

2.1.2. Age and sex

Although congenital malignant melanoma of the uvea has been reported,[30] it is rarely encountered in children or young adults. In most series, the median age at diagnosis is about 55 years.[20] Raivio found uveal melanomas to be most frequent in women in the sixth decade of life, and in males in the seventh.[2] Patients older than 40 years of age at the time of treatment have a worse prognosis than younger patients.[20,31]

2.1.3. Genetics

Cutaneous melanoma is an inherited disease in about 10% of cases,[32] but for uveal melanoma only about 13 clusters occurring among relatives have been reported; sometimes involving more than 2 generations.[33] Inheritance is reported to be most likely autosomal dominant with partial expression or incomplete penetrance.[34] An inheritable form of cutaneous melanoma is known as the familial atypical multiple mole melanoma (FAMMM), B-K mole syndrome or the dysplastic nevus syndrome: family members of persons with large numbers of dysplastic nevi have a high risk of developing cutaneous melanoma. A percentage of these cases is sporadic rather than familial. It is unclear whether a risk relationship exists between sporadic and familial dysplastic nevus syndrome and ocular melanoma,[35] but has been suggested by a Dutch study.[36] However, heredity is clearly not a major determinant of uveal melanoma.

2.1.4. Hormonal factors

Several observations suggest that hormonal factors are involved in uveal melanoma. Higher incidence rates of uveal melanoma have been found in young women than in young men;[19,37] an increased risk has been found for ocular melanoma after use of estrogen substitutes and a decreased risk among women who had undergone ovariectomy.[37] A related observation is the finding that survival for women with uveal melanoma seems to be more favorable than for men.[20,31] Gynecologic cancers tend to be more common in female patients with uveal melanoma.[38] A recent study found a decreased risk for women, who had ever been pregnant: the largest difference was observed between nulliparous and parous women. No other reproductive factors, including use of oral contraceptives or postmenopausal estrogens appeared to be related to the risk for uveal melanoma in that study.[39] A larger case-control study of cutaneous melanoma and reproduction variables have shown inconsistent results.[40]

2.1.5. Occupational exposure

The only specific occupational exposure which has been linked to uveal melanoma is welding, as did exposure to UV.[26]

2.1.6 Nevi, ocular melanosis and neurofibromatosis

The annual rate of transformation to malignant melanoma is probably in the range of 1 per 5000 choroidal nevi each year. Although the absolute risk of choroidal melanoma in individuals with choroidal nevi is low, the risk may still be greater than that in individuals without choroidal nevi.[17] Ocular melanocytosis and oculodermal melanocytosis (Nevus of Ota) are rare typically congenital unilateral hyperpigmentations, involving the uvea, scleral canals and episclera and predispose to uveal melanoma.[17]

Neurofibromatosis is possibly associated with cutaneous[41] and choroidal melanoma.[42] Although it has been suggested that neurofibromatosis patients have an increased incidence of uveal melanoma,[43] the number of reports is small.[44]

2.1.7 Associated extraocular primary cancers

One study on 400 uveal melanoma patients revealed that the overall prevalence of cancers diagnosed in these patients was over two times greater than the expected prevalence; basal cell carcinomas were excluded. They indicated a link between cutaneous and uveal melanoma.[38] In contrast, another study on 407 uveal melanoma patients found no elevated risks for a history of other prior cancer.[45]

2.2 Melanoma of the Iris

Melanomas of the iris are rare and account for 3 to 12% of uveal melanomas. Contrary to the relatively poor prognosis of malignant melanomas of the choroid and ciliary body, the vast majority of iris melanomas is known to consist of relatively benign, non-metastasizing lesions: only 37 cases of iris melanomas with presumed metastasis have been reported in the literature.[46] From a clinicopathologic study of 189 lesions it was concluded that most of these lesions are in fact biologically benign, "progressive," or "active" nevi, even though they might have produced a surface plaque growth onto the trabecular meshwork and peripheral cornea.[28] Fine needle aspirations of the iris are not helpful in differentiating nevi from small melanomas, but can be useful in differentiating melanocytic tumors from other benign tumors or metastatic lesions.

Conservatism is recommended in the management of iris tumors. Enucleation of an iris melanoma should be considered only if the tumor is too extensive (extrascleral growth, irreversible tumor-induced glaucoma) to be managed by other methods (local resection) or if the eye has no salvageable vision.[46]

2.3 Melanoma of the Choroid and Ciliary Body

In contrast to iris melanomas, tumors of the choroid and ciliary body pose a serious threat to life. The location of the anterior margin of the tumor, anterior to the equator of the eye is associated with an unfavorable outcome.[10] Most uveal melanomas arise from the choroid. The clinical symptomatology depends on the location of the tumor: tumors of the posterior pole cause early symptoms. They can be diagnosed by ophthalmoscopy and ultrasonography. Evidence of growth can be established by serial fundus photography. A recent clinicopathological study reported a clinical misdiagnosis rate of only 0.48%.[47] In clinical atypical cases fine needle aspiration biopsy, using a 22-gauge needle, can be reliable in differentiating amelanotic choroidal melanoma from choroidal metastasis and other amelanotic fundus lesions.[48] Relative contra-indications to fine-needle aspiration of a suspected intra-ocular tumor are 1) the presence of an intra-ocular mass in which the diagnosis is reasonably well established on the basis of ocular examination and non-invasive diagnostic studies 2) a small melanocytic choroidal or iris lesion in which the differential diagnosis is between a large nevus and a small melanoma and 3) the presence of a localized mass, for which local resection is contemplated.[48]

The diffuse, flat malignant melanoma of the choroid is rare, and characterized by diffuse insidious growth throughout the uvea. Clinical diagnosis of these tumors is difficult and usually made in later stages; diffuse, flat melanomas tend to have a particularly poor prognosis.

The growth of uveal melanoma may occur in three directions, i.e. outward (through the sclera), inward (through Bruch's membrane towards the vitreous) or in the uveal plane. Scleral invasion occurs along the scleral emissary veins and may be found very early in the course of the disease. Extrascleral growth is observed in between 3.8% and 40% of the cases.[31,49] Once Bruch's membrane has been ruptured, subsequent tumor growth is most extensive into the subretinal space. Bruch's membrane exerts an elastic restraining effect around the rupture, leading to a mushroom shaped mass in which blood outflow is impeded (Figure 1). Changes at the front of tumor invasion such as loosening of the cellular union and transformation to a more malignant cell type, has not been demonstrated light microscopically.[50]

Figure 1: A mushroom shaped malignant melanoma of the posterior uveal tract, breaking through Bruch's membrane (arrows). Hematoxylin/eosin, original magnification x 12.

2.4 Classification

Malignant melanomas have a spectrum of cell types, ranging from thin and plump spindle cells to epithelioid cells. In 1931 Callender developed a cytologic classification of uveal melanomas.[51] The following types of melanoma cells were recognized in the *original Callender classification.* 1) Spindle A cells are uniform, slender spindle-shaped cells, containing fusiform nuclei often showing a longitudinal streak (Figure 2a). 2) Spindle B cells are plumper spindle cells with larger ovoid nuclei, and conspicuous nucleoli (Figure 2b). Spindle cell tumors tend to grow in a compact cohesive fashion, and they generally have a dense framework of reticulin fibers. Callender defined a fascicular type melanoma as a tumor composed of spindle B cells arranged in columns, or fasciculi (Figure 2c), often orientated at right angles to a centrally located capillary, which is now classified as a spindle cell melanoma. 3) Epithelioid cells are much larger, polyhedral, sometimes very pleomorphic cells with abundant acidophilic cytoplasm, large pleomorphic nuclei and large acidophilic nucleoli (Figure 2d). These cells grow less cohesively than spindle cells and are not surrounded by a network of reticulin. Melanomas of the mixed cell type are composed of a mixture of epithelioid and spindle cells. Later another category, "necrotic" melanoma was introduced. In addition small epithelioid cells (Figure 2e) and large lipid- and/or glycogen containing tumor cells were recognized (Figure 2f). This classification has appeared not to be sufficiently reproducible.[52]

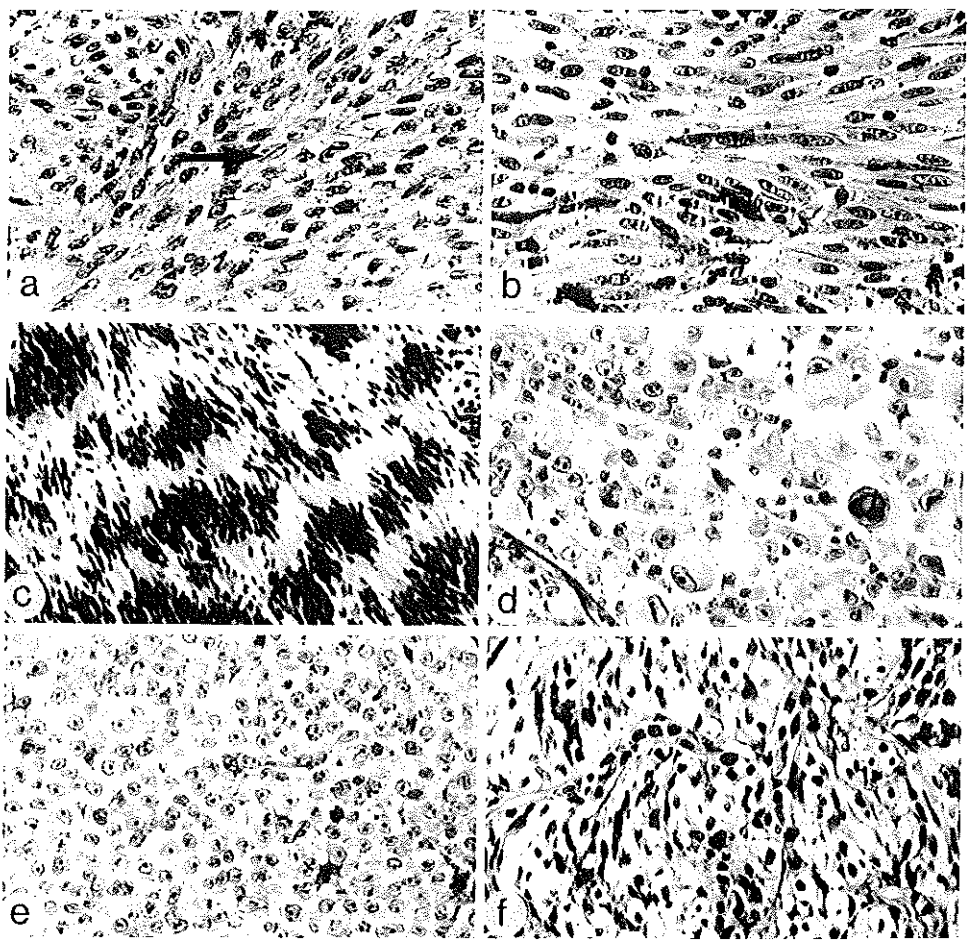

Figure 2: a) Spindle A cells, characterized by slender spindle-shaped cells, containing fusiform nuclei often showing a longitudinal streak (arrow). b) Spindle B cells: plumper spindle cells, with larger ovoid nuclei, and conspicuous nucleoli. c) Fascicular type melanoma, composed of spindle cells arranged in columns. d) Epithelioid cells: less cohesive, polyhedral pleomorphic cells with abundant acidophilic cytoplasm, large nuclei and large acidophilic nucleoli. e) Small epithelioid cells. f) Large clear cells: lipid and/or glycogen containing tumor cells. (a - f: Hematoxylin/eosin, original magnification x 361).

McLean et al. demonstrated that spindle cells include a spectrum of benign and malignant cells and they proposed a *modification of the Callender classification*: spindle cell melanomas, mixed cell melanomas and epithelioid cell melanomas.[53,54] This classification is currently applied. They also demonstrated that all melanomas containing epithelioid cells

had more than 50% chance of metastatic spread, but when the epithelioid cells were small the prognosis was not quite so bad. Although important trends have emerged from retrospective prognosis studies, such as relevance of size and cell type, Markowitz et al. noted the absence of information on study design and methods in 50% of 76 melanoma studies from 1966 to 1988.[55]

In *recent studies* of uveal melanomas the presence or absence of any epithelioid cells (spindle cell melanoma versus a combination of mixed cell type and epithelioid cell tumors) has been used.[31,56,57,58]

The biological behavior of uveal melanoma (tumor grade) depends not only on the cell type but also on several other factors including largest tumor dimension (LTD), especially LTD in contact with sclera,[10] the location of the tumor[10,59] and the presence or absence of extraocular extension. Tumors situated anteriorly are more associated with subsequent metastases than those located posteriorly. The leading predictors of survival are LTD and the presence of epithelioid cells:[10,58] LTD ≤12 mm and less than two epithelioid cells per high power field (HPF) is associated with a favorable outcome.[10] A meta-analysis of melanoma reports between 1966 and 1988 indicated that the combined weighted estimates of 5-year mortality rates following enucleation were 16% for small (LTD ≤10 mm) tumors, 32% for medium (LTD: 10-15 mm), and 53% for large (LTD >15 mm) tumors.[5] By univariate analysis cell type and LTD share a similar level of correlation with death from uveal melanoma, but only a limited correlation between cell type and LTD could be demonstrated.[57,58] Recent investigations have suggested that the presence of vascular networks, defined as at least three back to back closed vascular loops (Figure 3), is a feature strongly associated with death from metastatic melanoma.[60,61]

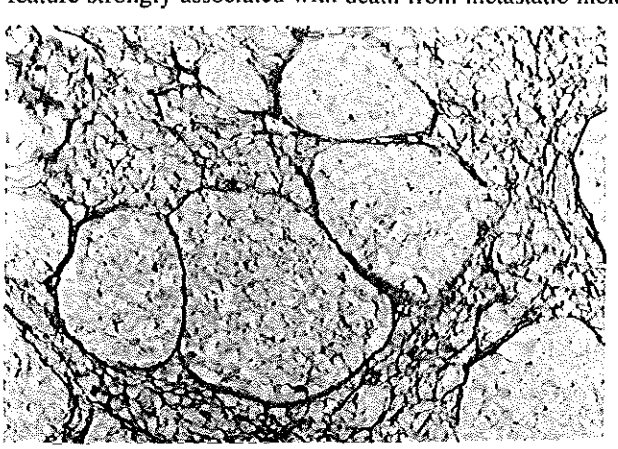

Figure 3: Closed vascular networks in uveal melanoma. Periodic-acid Schiff, without hematoxylin/eosin counterstaining, magnification x 361).

Other significant factors in the Cox proportional hazard model included in that study (in descending order of importance) LTD, mitoses, the parallel with cross-linking vascular pattern, age, the presence of tumor-infiltrating lymphocytes (TIL),[60] and male gender.[31,60] Cell type and tumor location did not appear to be an important prognostic parameter in the stepwise modelling in that study.

Tumor stage indicates the extent of spread from the primary site: this can be recorded according to the pTNM pathologic classification,[62] which is shown in Table 1.

Table 1 *TNM Classification of malignant melanoma of the posterior uvea*

Pre-treatment Clinical Classification

T - Primary Tumor: ***Ciliary body***

T1	Tumor limited to the ciliary body
T2	Extension into the anterior chamber and/or iris
T3	Extension into choroid
T4	Extra-ocular extension

T - Primary Tumor: ***Choroid***

T1*	≤10 mm LTD *and* ≤3 mm elevation
	T1a: ≤7 mm LTD *and* ≤2 mm elevation
	T1b: > 7-10 mm LTD *and* > 2-3 mm elevation
T2*	> 10-15 mm LTD *and* > 3-5 mm elevation
T3*	> 15 mm LTD *or* 5 mm elevation
T4	Extra-ocular extension

N - Regional Lymphnodes

N0	No regional lymphnode involvement
N1	Evidence of regional lymphnode involvement

M - Distant metastases

M0	No evidence of distant metastases
M1	Evidence of distant metastases

*: When LTD and elevation show a difference in classification, the highest category should be used for classification.

Table 1 (continued) *TNM Classification of malignant melanoma of the posterior uvea*

P.TNM Post-surgical Histopathological Classification

pT- Primary Tumor: ***Ciliary Body and Choroid***

G - Histopathologic Grading

G1	Spindle cell melanoma
G2	Mixed cell melanoma
G3	Epithelioid melanoma
GX	Grade can not be assessed

S - Scleral invasion

S0	No evidence of scleral invasion
S1	Evidence of intrascleral invasion
S2	Evidence of extrascleral invasion

V - Venous Invasion

V1	Veins in melanoma contain tumor
V2	Vortex veins contain tumor
VX	Venous invasion can not be assessed

pN and pM categories correspond to clinical N and M categories

STAGE GROUPING: Ciliary Body and Choroid.

Stage I	T1 N0 M0
Stage II	T2 N0 M0
Stage III	T3 N0 M0
Stage IVa	T4 N0 M0
Stage IVb	any T N1 M0; any T any N M1

This classification combines tumor size (LTD, tumor height, presence or absence of extrascleral growth)(T), cell type (G), spread to regional lymphnodes (N) and presence or absence of metastases (M). The practical value of this classification for uveal melanoma is limited: uveal melanomas do not metastasize to regional lymphnodes, and the presence of clinically detectable metastasis at the time of diagnosis of the primary tumor is rare. Furthermore LTD is in most studies a more important prognostic factor compared to the presence of extrascleral growth; tumor height seems of minor importance in this respect. Combining these factors does not provide additional value.[63]

2.5 Survival of Choroidal Melanoma Patients

In a 7 1/2 year follow-up study from a series of 234 patients treated in Rotterdam between 1971 and 1990 the survival rate was 74%.[64] These survival curves are similar to recently published survival curves.[31,59] Survival for women with choroidal melanoma was more favorable than for men.[64] This is concordant to the findings of Folberg et al.[31] and Jensen.[20] Unfortunately a proportion of patients with relatively favorable tumor characteristics, like small size and pure spindle cell composition, still develops metastases. Survival data indicated that uveal melanoma create a peak incidence of mortality during the second and third years following enucleation, irrespective of LTD. Therefore it has been argued that surgical manipulation of the eye during enucleation could mechanically squeeze tumor cells into the blood stream, thus enhancing dissemination.[65] Proponents of this hypothesis point to the lack of detectable metastases when the patient first presents. However, in patients treated by enucleation tumor cells must have disseminated prior to or during treatment, probably due to micrometastases. Survival time for patients dying of metastatic disease is primarily a function of the growth rate of the tumor cells.[16] Survival curves for T1 tumors showed that the peak in death rate for these relatively small melanomas shifted to the fourth postoperative year, being half the size of the peak for T4 tumors.[63] Manschot postulated that -based on calculated doubling times (Td's) of skin melanomas- Td's of uveal melanomas with a significant epithelioid cell component might vary between 30 and 100 days, and Td's of spindle cell melanomas between 100 and 365 days.[16,66] In a review of 39 published calculated doubling times of uveal melanomas 36 appeared to be longer than 60 days. Metastatic death occurred 35-40 Td's after dissemination. The shortest interval between dissemination and metastatic death in individual patients was therefore calculated as 35 x 60 days = 6 years. It was postulated that all metastatic deaths within these 6 years are due to pretreatment dissemination.[67] Our inability to predict survival with a high degree of accuracy is due to a lack of understanding of the factors which initiate and control the metastatic process in uveal melanomas.

2.6 Therapeutic Modalities

The management of malignant melanoma of the posterior uvea is still controversial. Small asymptomatic choroidal melanomas can probably be observed periodically until evidence of growth is documented. It is still generally agreed that enucleation is necessary for large

choroidal and ciliary body melanomas, and for melanomas that have caused glaucoma[68,69,70] Pre-enucleation radiotherapy has been employed in some centers, although recent doubts have been raised to its effectiveness in preventing metastasis.[64,71,72] Other modes of treatment include local resection for melanomas located nasally and those located more than one disc diameter from either the optic disc or fovea,[73] laser photocoagulation for some small choroidal melanomas,[74] and radiotherapy.[69]

Currently, radiotherapy is the most widely advocated treatment modality for medium-sized melanomas by some authors.[69,70] The most commonly used type of radiotherapy has been the episcleral application of a radioactive plaque. Iodine, Cobalt, Iridium, and Ruthenium plaques are being used for this purpose. Another method of radiotherapy is charged particle irradiation.[75] The two methods of radiotherapy seem to have equal results with regards to the development of systemic metastatic lesions.[69,70]

Orbital exenteration is sometimes necessary for advanced uveal melanomas with massive extraocular extension,[69,70] although it is questionable if this prolongs life expectancy.

Currently, the most frequently advocated treatment methods are enucleation and episcleral plaque brachytherapy. On the basis of currently available information, 5-year survival rates for patients who receive radiotherapy are similar to those for patients treated by enucleation.[76] Studies with limited follow-up have shown that 5 to 10% of patients who receive radiotherapy ultimately require enucleation of the affected eye because of recurrent tumor growth or radiation-induced complications.[77]

Manschot postulated that the shortest interval between dissemination and metastatic death may be calculated as 6 years. Therefore local therapy cannot influence the survival rate within the first seven post-therapeutic years.[67,78] An analysis of published, histopathologically studied irradiated melanomas revealed a retained reproductive integrity.[78] He postulated that the risk for post irradiation exponential growth cannot become manifest before a 10-year follow-up study, leaving few justifiable indications for radiotherapy.[78] A large collaborative study to evaluate the relative effectiveness of enucleation and iodine plaque radiotherapy is under way.[79]

Recently a reverse transcription/polymerase chain reaction amplification of the tyrosinase gen has been used to detect circulating tumor cells as first sign of dissemination from uveal melanoma.[80] This may be important when considering the administration of adjuvant therapy.

Currently no effective treatment exists for metastatic uveal melanoma. The effectiveness of new approaches for the management of metastases, involving interferons, interleukin, and

combination chemotherapy has not been determined. Intrahepatic administration of chemotherapy for hepatic involvement of metastatic uveal melanoma is under study. When an effective systemic treatment for metastatic uveal melanoma has been found, early administration as an adjuvant to primary treatment for patients with a high risk of developing metastasis may provide the best strategy for control of systemic spread.[81] Immunotherapy is under study.

References: page 37

CHAPTER 3: Prognostic Parameters in Uveal Melanoma: A Review

3.1 Morphometry and DNA Analysis

The interobserver error of the conventional Callender classification has proven to be large.[52,82] The original Callender system was simplified in order to reduce the number of categories, which has improved the correlation of histologic features with malignant behavior.[54] However, since morphology varies as a continuum, morphological classification schemes are inherently arbitrary and subject to variations in interpretation.[59] Subsequently, a semi-quantitative system was developed to estimate the percentage of epithelioid cells in each tumor[10] and the number of epithelioid cells and inverse standard deviation of the nucleolar area.[83] This refined method still remained subjective. Furthermore, it has been shown that measurements of LTD made from glass microslides correlate with direct measurements taken from the cut surface of the globe at the time of gross examination, if the eye was cut to obtain representative sections.[84]

Methods have been devised to measure cytologic features objectively. One of the early methods developed was measuring the standard deviation of nucleolar and nuclear features, which confirmed the value of nuclear pleomorphism for predicting the malignant potential of uveal melanomas.[85] The standard deviation of the *nucleolar* area (SDNA) proved to be a useful measure of the malignancy of intra-ocular melanomas, especially when coupled with LTD.[86,87] The standard deviation of both nucleolar area and circumference correlated highly with survival: the nucleolar area was usually greater and more pleomorphic in the more malignant type of uveal melanoma (epithelioid or mixed versus spindle cell tumors). Unfortunately this method requires an elaborate specially designed system. A simpler system was developed to calculate the mean of the diameters of the ten largest nucleoli (MLN) encountered in a strip 5 mm long and one oil immersion field wide through the center of the tumor.[87,88]

A comparison of prognostic covariables for uveal melanoma revealed that LTD, Callender cell type, SDNA and MLN correlated equally with tumor related death. MLN can be measured more easily than SDNA and is more reproducible than cell type and is therefore a useful prognostic parameter.[89] Multivariate analysis revealed that adding LTD as a prognostic covariate to either cell type, SDNA, or MLN yields a substantial increase in prognostic value.[6,89] A recent study demonstrated that, using multiple Cox regression models, MLN failed to exert any effect on outcome after enucleation. These authors found the presence of closed vascular loops to be the most statistically dominant histologic

prognostic characteristic.[90]

A morphometric analysis studying *nuclear* as well as nucleolar features revealed that the standard deviation of nuclear area was the most significant variable, followed by the difference between nuclear and nucleolar areas, SDNA.[91] A combination of (tumor-induced) glaucoma and standard deviation of nuclear area appeared to be the most significant prognostic combination.[58,91] Recently syntactic structure analysis has been applied to uveal melanomas. This is a mathematical method describing the basic structural units such as cells, glands and vessels as a network.[91] This technique may be able to differentiate between various Callender cell types.[91]

Nucleoli are sites of active RNA synthesis, and a relationship between nuclear RNA content and prognosis was investigated by flow cytometry using acridine orange, a metachromatic fluorochrome: the nuclear RNA content appeared to be an independent prognostic indicator in uveal melanomas, and increased RNA content was associated with a worse prognosis.[92]

Nucleolar organizer regions (NORs) are outpouchings of nucleolar DNA, that direct ribosomal RNA transcription. Silver staining of the nucleolar organizer regions (AgNORs), visualizes the size and number of nucleolar organizer regions as black dots, seemingly reflecting or predicting cellular proliferation. Application of this method to a series of uveal melanomas disclosed a correlation of AgNOR counts with mitoses and tumor size; however, the prognostic value of AgNORs remains to be established.[93]

DNA flow cytometry has shown to be a reliable quantitative method of determining the nuclear DNA content (ploidy) of a tumor cell sample. For uveal melanoma it has been shown that ploidy abnormalities correlate more closely with survival than the standard histologic parameters.[94,95]

DNA quantitation can also be carried out by static image analysis, usually by measuring the amount of light transmitted through tumor cell nuclei in a Feulgen stained specimen. For uveal melanomas it has been found that all spindle A cells are diploid and most tetraploid peaks are formed by epithelioid cells.[96] This method, however, does not appear to have additional prognostic value.[97]

Bromodeoxyuridine, a thymidine analogue, is incorporated during the synthesis phase of the cell cycle. Measurements of DNA synthesis by bromodeoxyuridine incorporation in uveal melanoma showed destruction of reproductive integrity of melanomas, which received 20 Gy pre-enucleation radiation.[98]

3.2 Genetic Abnormalities

3.2.1. Cytogenetics

The association of consistent chromosomal aberrations with particular types of cancer has led to the identification of some of these genes and the elucidation of their mechanism of action. The common tumor chromosome aberrations are generally classified as structural or numerical. Structural alterations include translocations, inversions, deletions, insertions, and amplifications, whereas numerical abnormalities are losses or duplications of whole chromosomes.

For cutaneous malignant melanoma and their cell lines, frequent involvement of chromosomes 1, 6, 7, 9 and 11 in structural aberrations has been reported.[99-103] It has been suggested that chromosome 9 plays a role during the development of cutaneous malignant melanoma, whereas chromosomes 2,3 and 6 are most likely associated with progression.[104]

Cytogenetic studies of uveal melanomas revealed that monosomy of chromosome 3 and multiplication of chromosome 8q do not occur randomly in uveal melanoma.[105-111] Monosomy 3 and gain of 8q have exclusively been demonstrated in ciliary body melanomas,[108,109] and may therefore be associated with a subgroup of uveal melanomas with poor prognosis.[107] Furthermore aberrations of chromosome 6 (loss or gain) have been described,[106,107,112] In cell lines obtained from metastatic uveal melanoma no consistent chromosomal aberrations have been demonstrated.[113]

3.2.2 Oncogene activation

The genetic damage found in cancer cells comprises two different categories 1) dominant mutations which can activate proto-oncogenes to become oncogenes, 2) recessive mutations in target genes known as tumor suppressor genes or anti-oncogenes. They are called recessive because both copies must be inactivated for tumor formation to occur.

It is generally accepted now that molecular alterations in oncogenes and tumor suppressor genes are responsible for the conversion of a normal cell into a tumor cell. Usually these genes exert normal functions in cell proliferation and differentiation.[15]

Little research has been carried out to identify and analyze the role of oncogenes and tumor suppressor genes in the development and prognosis of choroidal melanoma. Several oncogenes, including c-myc, have been mapped to 8q.[103,114] The expression of c-myc oncoprotein has been investigated in uveal melanomas and compared with other prognostic factors. Positive staining for c-myc protein correlated with proliferative index in diploid tumors, and with HMB-45 staining, but not with cell type.[115] In a study on prognosis, the

percentage of c-myc positive cells was strongly associated with tumor-related death. Furthermore a strong correlation between c-myc and the Mib-1 defined proliferative index was found.[116]

Point mutations in the N-*ras* gene occur in cutaneous malignant melanomas. In patients with the N-*ras* mutation, sun exposure could have been the etiologic agent of these melanomas.[117] For uveal melanomas no N-*ras* mutations were detected in any uveal melanoma studied.[118,119]

The expression of the oncoproteins c-neu (c-erb-B2: epidermal growth factor receptor) and *ras* were also analyzed with specific MAbs in a small series of uveal melanomas: in one metastasizing melanoma marked expression of *ras* was obvious.[120]

Recent evidence suggests that mutation of the p53 suppressor gene (named for the protein it encodes, p53) is one of the most common abnormalities found in human cancers.[121] This gene is located on the short arm of chromosome 17 and encodes a 53 kD nuclear protein that appears to be involved in regulating the cell cycle. The normal p53 product has been shown to act as a tumor suppressor, but various point mutations within the coding region of the gene inactivate or alter this function. These mutations arise relatively late in neoplastic progression and may correlate with malignant transformation. However, to evaluate p53 as a diagnostic marker correlations between p53 mutation (DNA sequence), protein overexpression (immunocytochemistry positivity), and tumor behavior need to be considered.[122] For cutaneous melanoma significantly increased prevalence of mutant p53 was found in metastatic melanoma, compared with primary tumors.[123] In contrast, other investigators found that the p53 staining was not correlated to subsequent development of local metastases and a significant decrease of p53 protein in metastatic lesions was found, as compared with the corresponding primary tumors.[124] Staining for mutant p53 protein expression was found in 12 out of 18 uveal melanomas, whereas choroidal nevi were negative. In two melanomas expression of the p53 protein was confirmed by the demonstration of mutations in exon 7. These observations suggest that acquisition of abnormalities of the p53 gene may be an important step in the progression of uveal melanoma.[125] Recently, mutant p53 has been demonstrated in museum specimens of a family with a history of four generations of uveal melanoma associated with breast cancer.[126]

Recently, homozygous deletions of a gene (Multiple Tumor Suppressor 1) have been reported in a wide variety of tumors, including cutaneous melanomas. This gene encodes a protein, previously identified as inhibitor (p16) of an enzyme called cyclin-dependent

kinase 4 (Cdk4), which is involved in the cell division cycle.[127] Currently, uveal melanoma cell lines are under study.

Examination of mRNA levels of 21 different oncogenes, anti-oncogenes, growth factors and proteases in 17 cutaneous melanoma cell lines revealed a significant correlation between c-myc, p53 and c-src-1 (tyrosinase-kinase) levels, and between p53 and c-erb-B2 (EGF-receptor), which may be due to common regulatory control of these genes in cells of the melanocytic lineage.[128] This is in accordance with the view that oncogenic transformation is a multistep process, involving activation of at least two genes.[129]

So far no single gene has been shown to participate in the development of human cancers. For uveal melanoma, research in the field of molecular genetics has been limited. Further identification of tumor suppressor genes or oncogenes that participate in the cell cycle of uveal melanoma seems necessary.

3.3 Immunology

Cutaneous and uveal melanomas are considered to be relatively susceptible to immunologic influences because of reports of spontaneous regression,[130] and because of the delayed appearance of metastatic disease, sometimes decades after enucleation.[131] Five to 12% of uveal melanomas have tumor-infiltrating lymphocytes (TIL).[132] The number of cells is variable (0.1-29%), but usually low (mean 4,5%).[133] Immunohistochemical analysis of TIL revealed that T cells predominate in 74% of these cases, usually scattered, whereas B cells constitute a minority, usually present in clumps.[134] This was similar to the findings by flow cytometric analysis of TIL.[132] Remarkably, the presence of more than 100 TIL per 20 HPF in uveal melanoma is associated with a decreased survival rate.[31,135] T-lymphocytic infiltration was associated with death due to metastasis.[134] This is the opposite of what is observed with most solid tumors in adults, where the presence of TIL is associated with increased survival. It has been speculated that dissemination of tumor cells is required for the generation of a T lymphocyte-mediated immune response. Because the eye lacks lymphatic drainage the primary antigen processing is in the spleen.[132] Without lymphatic drainage uveal melanomas may be less likely to disseminate than most neoplasms.[6,134]

Expression of the histocompatibility antigens HLA I and II on neoplastic cells may be important in the host-tumor interaction. HLA A,B,C antigen are detectable in most primary cutaneous melanomas and HLA-DR expression is associated with increased thickness and early metastasis.[136] In cutaneous melanoma, class I and II antigen expression appeared more

pronounced in the presence of a lymphocytic infiltrate.[137]

3.4 Melanoma Associated Antigens

Immunohistochemical study is now commonly used by pathologists in the differential diagnosis of anaplastic tumors. Several MAbs recognizing melanoma associated antigens (MAA) in routinely fixed, paraffin-embedded tissue are currently used (Table 1). With antisera generated against the S100 protein, immunoreactivity was noted in 90 - 97% of primary choroidal melanomas.[138,139] In contrast, only 15% of the tumors stained with a MAb specific for both S100α and S100ß, whereas 85% of all cutaneous melanomas reacted with this antibody. These findings suggest the possibility that a variant S100 protein exists in choroidal melanoma.[138] This has been confirmed at the mRNA level by a quantitative PCR method: choroidal melanomas expressed little or no S100ß.[140] Although quite sensitive, antibodies to S100 are not melanoma-specific.

The monoclonal antibody HMB-45 labels a cytoplasmic antigen produced by fetal melanocytes and melanoma cells of adults.[141] It is specific and fairly sensitive for cutaneous melanoma and junctional nevi in formalin-fixed, paraffin-embedded tissue.[142] HMB-45 recognizes an antigen that is expressed by stimulated melanocytes.[143] More than 95% of choroidal melanomas express the HMB-45 antigen (Table 1).[138,139,144] The HMB-45 antigen is immuno-electronmicroscopically found in melanosomes at stage II and III. This leads to the conclusion that proliferating melanocytes express the antigen.[144] Benign proliferative cells cannot be distinguished from malignant proliferating cells, with the aid of this antibody. Burnier et al. found that expression of HMB-45 appeared to be greater in active uveal nevi, than in inactive nevi.[139]

MAb NKI-C3 (gp90-34) was positive in 81% of choroidal melanomas.[145] The antigens recognized by MAb NKI-beteb (gp 100) are localized at the inside of premelanosomal vesicles.[146] This MAb has a striking similarity to HMB-45 (which also recognizes gp 100), but in addition recognizes resting adult melanocytes in skin.[147]

Interestingly, MAb CAM 5.2 to cytokeratins (CK) 8 and 18 reacted with formalin-fixed, paraffin-embedded primary uveal melanomas (38%), more often than with their cutaneous counterpart (6-14%).[56]

Several other MAbs against uveal and cutaneous MAAs have been assessed on choroidal melanomas (Table 1).[148-150]

Many primary malignant tumors, including uveal and cutaneous melanomas, that initially

appear homogeneous by conventional light microscopy actually consist of heterogeneous groups of cells displaying different biochemical or antigenic properties. A light microscopic feature of some uveal melanomas is the presence of well-localized, morphologically distinct areas within the same tumor. These have been attributed to separate clones of cells in different growth phases. Such cells might be expected to have different and distinct antigenic and cytomorphologic characteristics. MAb MAb8-1H (Table 1) bound selectively to these morphologically distinct areas, which appeared to have differences in mean nuclear area by cytomorphometric analysis.[149]

In order to evaluate the differences and similarities in antigenic expression patterns between cutaneous and uveal melanomas, a panel of MAbs directed against cutaneous MAAs has been applied on (frozen sections) of choroidal melanomas. Table 2 represents the markers expressed by the majority of uveal melanomas. MAbs NKI/C3 and NKI/beteb had a high sensitivity for choroidal melanomas in frozen sections.[145,151,152] Although marked variation of antigen expression in uveal melanoma was noted, certain patterns of antigen expression within individual lesion were present.[151] A number of MAbs (NKI-beteb, NKI-C3, M-2-2-4, Pal-M1[153] and G7E2) stained uveal melanomas as well as cutaneous melanomas (Table 2).[152] Of a panel of 18 MAA-MAbs applied on uveal melanomas, the expression of nine MAbs (TP39.1, TP36.1, 345.134, M111, CL203.4, M2590) was similar to that in cutaneous melanomas.[154] The studies revealed that the high molecular weight (HMW) antigen and ganglioside antigens were markedly less expressed on uveal melanomas than on cutaneous melanomas.[151,152,154]

The prognostic value of these markers remains to be established, although preliminary findings indicate that expression of NKI-beteb is related to a favorable prognosis in uveal melanoma.[155]

HLA class I stained 75%-85% of the uveal melanomas.[133,152,154] No correlation has been found between HLA class I expression and uveal melanoma cell type.[133,156] Others observed an absence of class I expression on pure spindle melanoma,[154] or in contrast, expression of HLA class I to predominantly spindle cells.[152] HLA Class II was detected in a limited number of lesions.[133,152,154,156] A relationship between HLA class I or II with metastatic potential of uveal melanoma has, however, not been established. Uveal melanoma, that had been irradiated with 2x 4 Gy before enucleation, had a significantly lower lymphocytic infiltrate, and a significantly lower expression of HLA class II.[156]

It was concluded that the overall surface antigen phenotype of the uveal melanomas tested, differs markedly from that of cutaneous melanomas as defined by the panel of MAbs

directed against cutaneous MAAs.[152] This might suggest a different origin of the normal melanocyte giving rise to uveal tumors.[152]

Table 1 *Melanoma-associated antibodies expressed on choroidal melanomas, assessed on paraffin embedded tissue*

MAb	Specificity	% les.	% cells	Reference
S-100	monoclonal	90%	5-100%	Burnier[139]
		20%*	>75%*	Burnier[139]
S-100	polyclonal	97%	n.s.	Kan-Mitchell[138]
MAb-079	S100α/ß	15%	n.s.	Kan-Mitchell[138]
HMB-45	100 kD	96%	n.s.	Kan-Mitchell[138]
HMB-45	100 kD	100%	5-100%	Burnier[139]
		95%*	>75%*	Burnier[139]
HMB-45	100 kD	99%	5-100%	Steuhl[144]
		77%*	>50%*	Steuhl[144]
NKI-C3	gp90-34	81%	>10%	Ringens[145]
ME491	HMW	87%	5-100%	Folberg[148]
MAb8-1H	40-50 kD/O-MAA	n.s.	n.s.	Donoso[149]
4A3	55-60 kD/O-MAA	n.s.	n.s.	Damato[150]

*MAb: monoclonal antibody. % les.: percentage positive stained lesions; % cells: percentage positive cells. n.s.: not specified. *: sensitivity. kD: kilodalton. HMW: high molecular weight. O-MAA: ocular melanoma-associated antigen.*

Table 2 *Markers expressed on the majority of choroidal melanoma as recognized by MAbs raised against cutaneous melanomas*

MAb	Specificity	% les.	% cells	Reference
NKI-beteb	100 kD	86%	>50%	v.d.Pol[151]
NKI-beteb	100 kD	100%	>70%	Carrel[152]
NKI-beteb	100 kD	100%	>60%	Ringens[145]
NKI-C3	gp90-34	100%	>60%	Ringens[145]
NKI-C3	gp90-34	87%	-100%	Carrel[152]
Pal-M1	tr. rec.	n.s	<70%	v.d.Pol[151]
Pal-M1	tr. rec.	75%	20-80%	Carrel[152]
M-2-2-4	diff. ag.	37%	5-100%	v.d.Pol[151]
M-2-2-4	diff. ag.	87%	>80%	Carrel[152]
G7E2	gp120-110	87%	30-100%	Carrel[152]
225.28S	HMW	n.s.	<70%	v.d.Pol[151]
225.28S	HMW	50%	<50%	Natali[154]
G7A5	HMW	75%	>80%	Carrel[152]
AMF-6	HMW	n.s.	n.s.	v.d.Pol[151]
AMF-6	HMW	50%	>70%	Carrel[152]
AMF-6	HMW	17%	n.s.	Ringens[145]
AMF-7	MA-CAM-1	17%	5-100%	v.d.Pol[151]
AMF-7	MA-CAM-1	63%	-100%	Carrel[152]
CL 203.4	ICAM-1	87%	>50%	v.d.Pol[151]
CL 203.4	ICAM-1	42%	5-100%	Natali[154]
Pal-M2	gp-95	75%	20-80%	Carrel[152]
Mel 14	gp33-38	68%	5-100%	Carrel[152]
Me14/D12	gp33-38	68%	5-100%	Carrel[152]
R24	GD3	50%	5-100%	Carrel[152]

MAb: monoclonal antibody. % les.: percentage positive stained lesions; % cells: percentage positive cells. kD: kilodalton. gp: glycoprotein. Tr. rec.: transferrin receptor. Diff. ag.: differentiation antigen on melanomas and nevi. HMW: high molecular weight. MA-CAM: melanoma-associated cellular adhesion molecule. ICAM: intercellular adhesion molecule. n.s: not specified.

3.5 Cell-Cell and Cell-Matrix Interaction

3.5.1. Cell-cell interaction

Cell adhesion molecules (CAM) are transmembrane proteins, connecting the cytoskeleton with the extracellular matrix. CAMs have been implicated not only in intercellular recognition, but also in morphogenetic events, regeneration, tumor invasion and metastasis. One CAM binds either to another identical CAM molecule on an opposing cell (homophilic binding) or binds to a receptor molecule of different identity (heterophilic binding). Of these adhesion molecules four families can be recognized: the immunoglobulin superfamily, the cadherin family, the integrin superfamily and the selectin family.[157,158]

1) The immunoglobulin (Ig) superfamily contains a series of CAMs which mediate Ca^{2+}-independent cell adhesion. Most members of this family are single-path transmembrane glycoproteins, which act by homophilic recognition. Several subfamilies can be distinguished: i.e. the neural cell adhesion molecule (NCAM) and the carcino-embryonic antigen (CEA) subfamily. The intercellular adhesion molecules (ICAM), vascular adhesion molecule (VCAM) and leucocyte function-associated antigens (LFA) also belong to this family.[157]

2) The cadherins are Ca^{2+}-dependant CAMs, which bind cells by means of homophilic interaction. Among the better characterized cadherins are: E-cadherin (also known as L-CAM), N-cadherin and P-cadherin. Each of the cadherins displays a unique pattern of tissue distribution.[157]

3) The integrin superfamily are mediators of cell-cell and cell-extracellular matrix adhesion. Integrins are divided into subfamilies, each with a common ß-subunit capable of associating with a group of α-subunits. The α- and ß-subunits in various combinations form at least 20 different types of integrins. The $ß_1$ (also known as very late activation or VLA-antigens) integrins comprise a subfamily in which eight α chains combine with one ß (the $ß_1$) chain. The vitronectin receptors share a common αv chain.[158]

4) The selectins are adhesion-receptor glycoproteins with an EGF-like domain. They mediate the migration of neutrophilic granulocytes in developing inflammatory reactions and are found on endothelial cells and leukocytes (ELAM).[157]

Cell surface receptors which mediate cell-cell and cell-matrix interaction in processes like metastasis, have been the subject of intense investigation during the past decade. However, studies on uveal melanomas are few and so far limited to the Ig-superfamily and the integrins.

For cutaneous melanomas ICAM-1 and a related melanoma-associated CAM (MUC 18) have been studied in the context of metastatic behavior.[159,160] Some investigators found expression correlated with metastatic behavior[159] but others demonstrated that these adhesion molecules occur on a full range of benign and malignant melanocytic lesions.[160] In uveal melanomas, cell adhesion molecule ICAM-1 could not be detected[152] and melanoma-associated CAM AMF-7 was not[145] or poorly expressed[151,152] (Table 2) compared to cutaneous melanomas. Others found ICAM-1 on most of the uveal melanomas[151,154] (Table 2) preferentially on the mixed and epithelioid cell type.[154] For cutaneous melanomas it has been suggested that loss of VCAM-1 may be important in the development of metastases.[160] VLA-2 (integrin $\alpha 2\beta 1$)[161] and the vitronectin receptor ($\alpha v\beta 3$)[162,163] have been shown to be preferentially expressed in vertical growth phase of primary cutaneous melanoma lesions and metastases, suggesting a role in melanoma progression. In contrast to cutaneous melanomas, expression of the $\alpha 2$ integrin was rare in uveal melanomas, and $\alpha 5$ expression was found in all lesions.[164,165] Furthermore, $\alpha v\beta 3$ was not detected in any of the primary uveal melanomas, but in two out of four metastases.[164] All primary lesions strongly expressed $\alpha v\beta 5$.[164,165] In contrast to cutaneous melanoma, it seems that determination of the integrin expression profile is not suitable for categorizing uveal melanomas as less malignant and highly malignant lesions.

3.5.2. Cell-matrix interaction

During several steps of tumor development, proteolytic degradation of the extracellular matrix and other tissue barriers is required.[166] Tumor- or host-derived proteinases are major participants in this process. Different proteolytic enzyme systems are involved, including the matrix metalloprotease system[167] and the plasminogen activator-plasmin system.[168] For cutaneous melanomas, it has been demonstrated that expression of plasminogen activators (urokinase- and tissue type plasminogen activators: u-PA and t-PA), their inhibitors, and urokinase receptors emerges in late stages of tumor progression.[169] Little is known about the role of proteases in the progression of uveal melanomas. An involvement of proteases in metastatic spread of uveal melanoma has recently been suggested by Cottam et al.,[170] who detected the 72 and 92 kD type IV collagenase in culture medium of 15 primary cultures of uveal melanomas. Furthermore, t-PA activity in supernatants of primary cultures of uveal melanoma seemed to correlate with scleral invasion in the tumor lesion, whereas no u-PA activity could be detected.[171] A histochemical study of uveal melanomas revealed that t-PA is markedly present at the invasive front, but no relation with tumor related death could be established. Expression of u-PA correlated with occurrence of

metastases. The involvement of the PA system in uveal melanomas differed from that in cutaneous melanomas.[172] It is conceivable that in uveal melanomas other proteolytic systems are involved in metastatic spread.

Several cytokines, secreted either by tumor cells, stromal cells or TILs, may act to enhance the invasive potential of tumor cells, leading to metastasis.[167] The most important cytokines are: tumor necrosis factor α (TNFα), interleukin-1 (IL-1), transforming growth factor α and ß (TGFα/ß) and epidermal growth factor (EGF). Cutaneous melanoma cell lines, when treated in vitro with TNFα[173] or IL-1[174] display enhanced metastasis upon injection in nude mice. Studies on uveal and cutaneous melanoma cell lines have shown that TGFß, used as a co-stimulant with TNFα or IL-1, selectively augments expression of the 92-kD type IV collagenase.[175] Detectable levels of TNFα have been found in one out of 16 uveal melanomas, occurring in one out of two patients who developed metastatic disease.[176]

The role of cell-cell and cell-matrix interactions, major elements in the acquisition of metastatic capacity of uveal melanoma, needs to be further elucidated.

Summary

Extensive research in the field of cytomorphometry yielded a substantial increase in prognostic value by investigating different aspects of the nucleoli in the cells of uveal melanoma. Furthermore DNA aneuploidy is reported to be a valuable prognostic marker. Aneuploidy occurs potentially in epithelioid melanomas, which have traditionally been regarded as having the greatest metastatic potential. The architecture of the microcirculation (the presence of networks of closed vascular loops) and LTD were found to be the most dominant histologic prognostic parameters. Unlike cytomorphometric techniques such as MLN and SDNA, which require specialized instrumentation, the detection of vascular patterns requires only a periodic acid-Schiff (PAS) without hematoxylin counterstaining stain and a green filter to enhance the visualization of these patterns. However, the reproducibility of this method remains to be established. Nevertheless, small or pure spindle cell tumors may also develop metastases. Several attempts have been made to identify characteristics in the uveal melanoma genotype and phenotype, which could be associated with the variation in metastatic potential. This search revealed considerable differences in cytogenetic findings between cutaneous and uveal melanomas.

Patients with malignant melanoma may develop a cellular and/or humoral immune response

to melanoma-associated antigens expressed by the tumor cells. Although uveal and cutaneous melanomas share a large number of surface molecules, the specific surface phenotype of melanoma-associated antigens differs markedly between uveal and cutaneous melanoma. Furthermore, differences between uveal and cutaneous melanomas have been found with regard to cell-cell interaction and the proteinase systems, involved in the degradation of extracellular matrix degradation. Some of these findings may be related to the different (stromal) origin of the uveal melanocytes, from which uveal melanomas develop.

Since early intraocular lesions are rarely available for research, in vitro models will play an important role in further investigations concerning uveal melanoma progression.

References: page 37

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CHAPTER 4

Proliferative Activity in Bilateral Paraneoplastic Melanocytic Proliferation and Bilateral Uveal Melanoma.

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Summary

An 83-year-old man with painless, bilateral visual loss developed multiple pigmented lesions on his penis, his buccal mucosa and the skin of his thorax. Bilateral multiple heavily pigmented areas were noted in his eyes. A bronchus carcinoma was detected. Postmortem histopathological examination of the eyes revealed bilateral diffuse uveal melanocytic hyperplasia (BDUMH), in addition to multiple pigmented melanocytomas and bilateral ciliary body malignant melanomas. Active proliferation of the benign uveal melanocytes was demonstrated by immunohistochemistry. Mucosal and cutaneous pigmentations have so far not been described as part of this paraneoplastic syndrome.

Introduction

Bilateral diffuse uveal melanocytic hyperplasia (BDUMH) is a rare but well known paraneoplastic syndrome,[1] associated with systemic malignancy. An essential feature of this syndrome is a preponderantly benign looking cytology of the melanocytic tumours.[2] We describe the clinical and histopathological features of BDUMH with active proliferation and bilateral ciliary body malignant melanomas. The multiple mucosal and skin pigmentations are an unusual feature of this syndrome.

Case Report

In 1990, an 82-year-old man presented with painless, bilateral visual loss for 3 weeks. He had been known to have a low grade non-Hodgkin's lymphoma for 6 years, which was in remission. Over one year he developed multiple pigmentations on his penis, the oral mucosa, and the skin, which were diagnosed as lentigo simplex.

Ophthalmic clinical evaluation revealed visual acuities of 0.7 in both eyes. Fundus examination revealed a heavily pigmented tumour in the posterior pole of both eyes (Figure 1). The peripheral part of both eyes contained multiple sharply defined, heavily pigmented areas. The clinical differential diagnosis included Gardner's syndrome and paraneoplastic syndrome.

In 1991 an bronchus carcinoma was detected, of which the patient died in 1992. Autopsy was not permitted; however permission was given to remove the eyes.

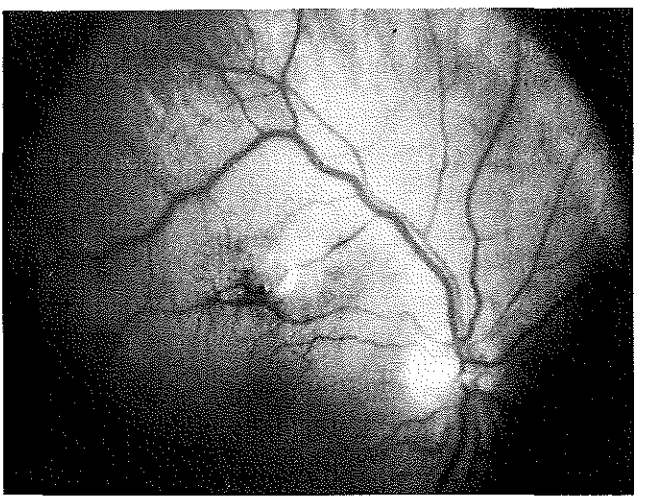

Figure 1. Fundus of the right eye, with a heavily pigmented tumour in the posterior pole, which on histological examination was consistent with melanocytoma.

On microscopic examination the choroid was diffusely infiltrated by plump spindle cells and small polyhedral cells. A few inconspicuous nucleoli were observed. Bleached sections of the heavily pigmented nodules showed a necrotic centre surrounded by large polyhedral cells with a basophilic central nucleus, compatible with melanocytoma cells. In both eyes the ciliary body was circumferentially thickened by atypical polyhedral cells with large nuclei, an irregular chromatin pattern and prominent nucleoli. A mitosis was sporadically observed. The tumour cells infiltrated the iris basis, the chamber angle, the trabecular system, and the pigment epithelium. Extrascleral, subconjunctival growth was noted in the right eye. In the macula focal depigmentations of the retinal pigment epithelium was observed, with atrophy of the photoreceptors.

On immunohistochemical examination the uveal melanocytes and the malignant melanoma cells stained strongly positive with HMB-45, S-100, and NKI-C3. There was weak staining with enolase and negative staining with Leu-7 and neurofilaments. Uveal melanocytes showed at different places positive nuclear staining with a proliferation associated nuclear antigen (Ki-67) (Figure2). The positive staining was observed in less than 1 % of the cells.

On flow cytometric analysis, both the benign melanocytic hyperplasia and the ciliary body melanoma were diploid.

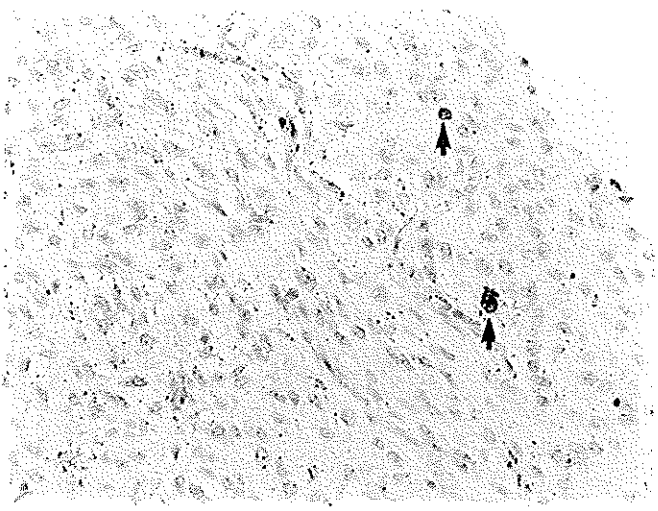

Figure 2. Photomicrograph of positive nuclear staining with Ki-67 (x361).

Comment

Paraneoplastic BDUMH is a rare, but well known paraneoplastic syndrome associated with systemic malignancy.[1] The primary tumour, when identified, has been found in varying organs. Our patient was known with a non-Hodgkin's lymphoma, which was in remission at the time of visual loss. In most patients described with BDUMH the visual complaints preceded the symptoms related to the primary tumour. The ocular and extra-ocular pigmentations in our patient developed before the clinical detection of the bronchus carcinoma.

Although a preponderantly benign appearing cytology of the melanocytic hyperplasia has been mentioned as an essential feature of this syndrome, bilateral multiple uveal melanomas occurred in 4 patients described.[3,4]

The strong expression of HMB-45 in the melanocytic hyperplasia of our patient is consistent with melanocytic activation.[5,6] Proliferation of the uveal melanocytes was focally demonstrated by a proliferation associated antigen. Both the benign melanocytic hyperplasia and the melanoma were diploid, which is consistent with relatively benign tumours. Active proliferation may have lead to malignant degeneration. The stimulus which leads to active

proliferation of preexisting anomalous melanocytes (that is,congenital uveal melanocytosis), is probably induced by a tumoral growth factor secreted by the primary tumour.[1] The visual loss of our patient can be explained by the depigmentation of the retinal pigment epithelium and the photoreceptor atrophy.

The combination of Ota/Ito's naevus and BDUMH has been described;[3] however, multiple extraocular lentiginous pigmentations have so far not been recognised as part of this rare paraneoplastic syndrome.

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CHAPTER 5

No N-*ras* Mutations in Human Uveal Melanoma: the Role of Ultraviolet Light Revisited

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Mutations in codon 12, 13 or 61 of the *ras* genes, H-*ras*, K-*ras* and N-*ras*, convert these genes into active oncogenes (Barbacid, 1987). *ras* Gene mutations can be found in a variety of tumour types, although the incidence varies greatly (Bos, 1989). In animals, a wide range of xenobiotic agents are capable of inducing mutations in the *ras* oncogene family (Barbacid, 1987). Little is known about the involvement of mutagenic agents in the induction of *ras* mutations in humans. In cutaneous melanomas mutations of the N-*ras* gene (codon 13 and 61) were found in seven out of 37 tested cases (Van 't Veer et al., 1989). The primary tumours of these 7 patients were exclusively located on continuously sun-exposed body sites. These mutations were all near dipyrimidine sites, suggesting an active role for ultraviolet (UV) radiation in the induction of the mutations. Shukla et al. (1989) found in one out of 22 tested primary cutaneous melanomas N-*ras* mutation in codon 61, but no details of sun exposure were available. Melanoma of the uvea (iris, ciliary body and choroid) is the most common primary intraocular malignancy in adults (Cutler & Young, 1975). The incidence of uveal melanoma in whites is eight times the incidence in blacks and threefold greater then in certain Asian groups (Hakulinen et al., 1987). In the Caucasian population individuals with light irides have three times the risk of developing uveal melanoma compared to persons with brown eyes (Gallagher et al., 1985). Early life exposures to sunlight have been found to be especially important in the development of intra-ocular melanoma. (Tucker et al., 1985). Recent epidemiological studies have reported an elevated risk for Northern European ancestry, light skin colour, the presence of 10 or more cutaneous naevi, use of sunlamps and intense sun exposure (Seddon et al., 1990). Holly et al. (1990) found an increased risk of developing uveal melanoma for the apparent effects of UV exposure (severe eye burn, snow blindness), and for host factors, like light eye colour and a propensity to burn rather than tan. These findings implicate sunlight as an environmental risk factor for this disease. The colour of the iris is determined by the degree of pigmentation; limited pigmentation leads to a blue or grey iris and high concentrations of melanin are present in brown irides. Melanin can absorb UV as well as visible light.

To investigate possible UV mediated activation of N-*ras* genes we analysed 29 uveal melanomas for mutations. Table 1 summarises the patient data. The location of the intra-ocular tumours is illustrated in Figure 1.

Table 1 *Patient characteristics*

Clinical characteristics:	No. of patients:
Sex	
male	17
female	12
Histology	
Spindle cell type	12
Mixed cell type	8
Epithelioid cell type	9
TNM classification*	
T1	2
T2	8
T3	15
T4	4
Pigmentation of the iris	
minimal	18
moderate	6
heavy	2
unknown	3

**TNM classification of ophthalmic tumours. UICC Geneva 1985.*

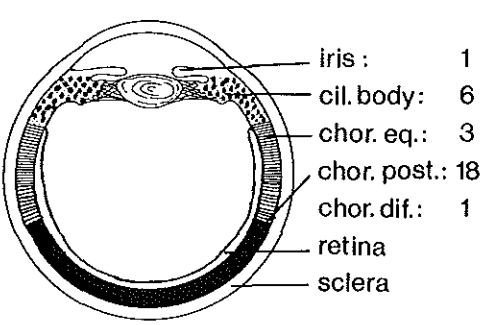

Figure 1: Location of the intra-ocular tumours. white area: iris. stippled area: ciliary body. striated area: equatorial choroid. black area: posterior choroid. diffuse (dif.) in the choroid: one melanoma.

When frozen tissue sections were examined it appeared that 17 samples contained 100%, 8 > 90%, 3 > 75% and 1 50% tumour tissue. DNA was extracted from five sections of 5 μm thickness of each tumour, adjacent to the one used for histopathology. The extracted DNA was used as a template in the polymerase chain reaction (PCR) following the protocol as supplied by the manufacturer of Taq polymerase (Cetus, USA). The primers used for the amplification of the fragment comprising codons 12 and 13 were sense primer 5' CTGAGTACAAACTGGTGGTTGGT 3', antisense primer 5'CAAAGTGGTTCTG-GATTAAGCT 3' and for amplification of codon 61: sense primer 5' AGTGGT-TATAGACGGTGAAAC 3', antisense primer 5'GTGCTCATGTATTGGTCTCTCAT 3'. The amplified fragments were separated from the primers on a low melting point agarose gel, eluted from the gel and subjected to an asymmetric amplification using a 200-fold lower concentration of the antisense primer (Gyllenstein, 1989). This results in preferential synthesis of the sense strands. The single strand was subsequently isolated from an agarose gel and used as a template in sequencing reactions using the antisense primers and Sequenase (Promega). The reactions were performed as suggested by the supplier of the enzyme, with a 4 fold higher concentration of dideoxynucleotides and an elongation step of 1 min. A plasmid with a mutation in codon 13 (GGT-GTT) served as a positive control, whereas normal DNA tissue served as a negative control.

Examples of autoradiographs from sequencing gels are depicted in Figure 2. No deviations from the wild type sequence were found in codons 12 and 13 in these samples, nor in the other 27 melanomas. Similarly, in none of the 29 melanoma samples mutations in codon 61 could be observed. It is clear from our study, that uveal melanomas differ from the cutaneous melanomas with regard to the N-*ras* mutation rate: N-*ras* mutations do not seem to play an important role in developing uveal melanoma. It is possible, however, that the other two *ras* genes may contain mutations. Shukla et al., (1989) described K-*ras* mutations in 3 out of 22 patients with primary cutaneous melanomas. In this latter study no correlation was found between *ras* mutations and UV exposure. The patients described here, were all Caucasians, lived in the Netherlands and possessed mainly light irides. This is consistent with some of the risk factors mentioned for developing uveal melanoma.

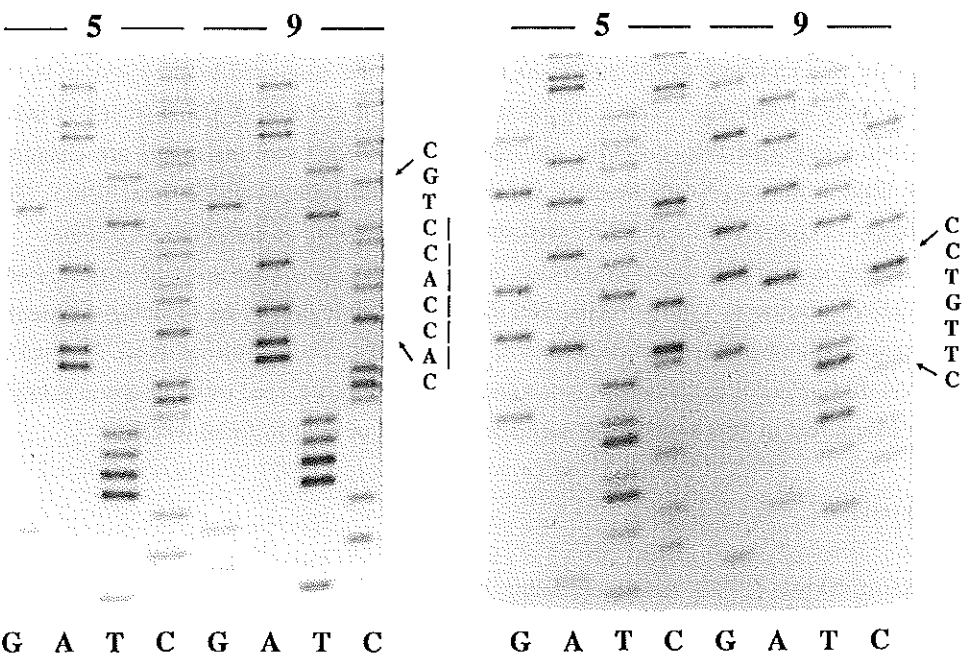

Figure 2: Example of autoradiograph from sequence gel. In the left panel the sequence of the amplified DNA from melanomas 5 and 9 is shown for the area surrounding codons 12 and 13. Note the sequence shown is that of the antisense strand. In the right panel the sequence reactions were performed on amplified DNA from the area surrounding codon 61 in melanoma 5 and 9 respectively (antisense strand).

The cornea effectively filters out all UV radiation shorter than 295 nm. In children a substantial transmission of UV-A (320-400 nm.) and UV-B (290-320 nm.) occurs, which decreases with age (Lerman, 1980). Short UV wavelengths (UV-B) cause the formation of pyrimidine dimers in the DNA (Kraemer et al., 1984). It is believed that the choroid and ciliary body are protected from UV exposure and also from a large portion of the more energetic wavelengths of the visible spectrum by the overlying retina and retinal pigment epithelium (Lerman, 1986). Thus we must conclude that although there is ample epidemiological evidence for a role of UV radiation as a risk factor in developing uveal melanoma, it is questionable if UV radiation is able to reach the choroid and ciliary body. In the contrary the iridic surface is not protected by the lens or by overlying tissue from UV A and B radiation. The well documented tendency for iris melanoma to occur in the inferior sector of the iris (Jacobiec et al., 1981), where exposure to sunlight is presumably

the greatest, supports the view that the origin of these tumours is environmentally related. However, the incidence of iris melanoma is much smaller compared to those arising in the ciliary body or choroid. Another argument against the direct role of UV radiation in uveal melanoma might be that incidence and mortality rates for uveal melanoma are changing very little in Europe, North America, Japan and Australia (Strickland & Lee, 1981). This finding is in contrast to the rapid increasment of the incidence of cutaneous melanoma.

Hersey et al.(1983) found an increase in T suppressor cells after solarium exposure and a relative decrease in T helper cells. Sunlight may work indirectly by inducing a systemic alteration in immunologic function (Stern, 1984). Although the role of these findings to human disease is not established, immunologic perturbations caused by exposure to sunlight may play a role in developing uveal melanoma, as part of multifactorial disease.

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CHAPTER 6

Ki-67 Immunostaining in Uveal Melanoma: the Effect of Pre-Enucleation Radiotherapy

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Summary

The reactivity of 33 choroidal and ciliary body melanomas with monoclonal antibody Ki-67, which recognizes a proliferation associated nuclear antigen, has been assessed and compared with clinicopathological parameters. In 23 cases, 8 Gy irradiation was given two days before enucleation. Nonirradiated melanomas had a significantly higher proliferation rate as defined by staining with monoclonal antibody Ki-67 as compared with irradiated tumors ($p=0.007$). Similarly, a strong relationship was found between pre-enucleation irradiation and low mitotic activity ($p=0.001$). There was no significant correlation between the presence of Ki-67 positive nuclei and histologic classification, largest tumor diameter, localization of the tumor, age, sex, scleral invasion, pigmentation, and lymphocytic infiltration. The relevance of Ki-67 immunohistochemistry for the assessment of the life prognosis of patients with uveal melanoma has to be studied prospectively.

Introduction

The monoclonal antibody Ki-67 reacts with a DNA associated[1] antigen in the nuclei at all phases of the cell cycle except the resting phase.[1-3] Immunocytochemical labeling with this antibody has been shown to correlate with generally accepted indices of cell proliferation such as autoradiography,[4] flow cytometry,[5] bromodeoxyuridine labeling index,[6] and thymidine labelling index[7] and therefore provides a simple and reliable means of rapidly evaluating the growth fraction of normal and neoplastic human cell populations. In some malignancies,[8-11] it has been shown that Ki-67 labeling can serve as a prognostic parameter and recently its potential use as monitor for therapy in hormone-dependent human prostatic carcinoma has been described.[12] We have used immunostaining with monoclonal antibody Ki-67 in a series of ciliary body and choroidal melanomas to assess the proliferative index and to investigate the effect of pre-enucleation irradiation. This was done by comparison of eyes that had been irradiated with 2x 4 Gy electron beam irradiation on the last two days before enucleation and nonirradiated eyes with ciliary body and choroidal melanoma. We also studied the possible relationship between Ki-67 immunostaining and various conventional clinical and pathological prognostic parameters.

Materials and Methods

Patient Selection

Specimens were obtained from 24 patients treated in Rotterdam, and from nine patients treated in Amsterdam between January 1987 and October 1988. As part of another study, melanoma patients in Rotterdam receive from 1978, local radiotherapy in two fractions of 4 Gy on the two days prior to enucleation. Thus, all eyes from Rotterdam, except one, were irradiated. The nine patients treated in Amsterdam were not irradiated. The enucleated eyes were transported immediately to the pathology department. After transillumination the eyes were cross-sectioned through the tumor and part of the tumor was snap frozen in OCT compound (Tissue-tek) and stored at -70°. The remainder of the eye was fixed in formalin and embedded in paraffin.

Histopathologic Examination

Conventional histologic sections stained with hematoxylin-eosin were prepared from the paraffin-embedded tissue. We determined in these the following parameters: cell type (spindle, mixed or epithelioid type), mitotic rate, largest tumor diameter, scleral invasion (absent; slight, <25% of the scleral thickness; moderate, approximately 50%; deep, >75%; episcleral growth), pigmentation (absent; slight, 25%; moderate, approximately 50%; heavy, >75%), and lymphocytic infiltration (absent, moderate or marked). Mitoses were counted in 15 consecutive high power fields with a total magnification of x400.

Immunohistochemistry

Frozen sections of 6 μm thickness were air dried and fixed for 10 minutes in acetone. Thereafter, slides were rinsed in phosphate-buffered saline (PBS, pH 7.4) and incubated with the monoclonal antibody Ki-67 (Dako Immunoglobulins Ltd, Copenhagen, Denmark). Slides were incubated with Ki-67 for 60 minutes at room temperature in PBS containing 0.01% gelatine and 0.1% sodium azide. As second-step reagent, a peroxidase-conjugated polyclonal rabbit anti-mouse immunoglobulin serum was applied (Dakopatts, Denmark). Subsequently, slides were rinsed in PBS to remove the unbound portion of the second reagent. After a final thorough washing in PBS, antigen-antibody binding was visualized by incubation in an acetate buffer solution (pH 4.6) that contained 3-amino-9-ethylcarbazole, dimethylformamide, and hydrogen peroxide.[13] Sections were counterstained with Mayer's hematoxylin for exactly 15 seconds to obtain a discrete nuclear staining pattern without

obscuring the Ki-67 reactivity. Positive reactions produced a red color, making differentiation with brown/black endogenous melanin pigment possible. Of all samples analyzed immunohistochemically with the Ki-67 antibody, consecutive frozen sections were stained routinely with hematoxylin-eosin for microscopic examination to evaluate the pathologic parameters.

As a negative control, specimens were stained with the second-step reagent only. Frozen sections of two primary and one metastatic cutaneous melanomas served as a positive control.

Assessment of the KI-67 Defined Proliferative Activity

Consecutive frozen hematoxylin-eosin sections of frozen tissue were examined by light microscopy. Counting of nuclei was performed at a magnification of x400. To facilitate the counting procedure a grid of measured dimensions was inserted in one of the ocular tubes. The number of nuclei was counted in three fields of an eye piece grid. Tissue sections stained with the antibody Ki-67 were examined and positive nuclei were counted in 15 eye piece grids and related to the amount of nuclei counted in the hematoxylin-eosin frozen section, thus measuring the percentage of positive cells in approximately 6000 nuclei in a random fashion: the number of high-power fields was randomly selected. Cell nuclei were considered to be positive if there was any nuclear staining present, regardless of the intensity and distribution within the nucleus. Counting of positive nuclei was repeated three times and averaged. Intra-observer variation varied between 0.02% and 0.25%. The Ki-67 defined proliferative activity or Ki-67 score was determined by the quotient of Ki-67 positive cells and total number of cells.[4]

Statistical Analysis

The non-parametric Mann-Whitney U test was used to determine the relation between pre-enucleation irradiation and Ki-67 proliferative activity. This test was also applied to study the relationship between pre-enucleation irradiation and level of mitotic activity. The Kruskal-Wallis test was used to examine the relationship between Ki-67 proliferative activity and histologic classification. For the relationship between the Ki-67 score and clinicopathologic parameters the Spearman Rank Correlation test was used.

Results

Immunostaining with Ki-67 was observed in all tumors tested, but the percentage of positively staining cells varied from case to case. Staining with monoclonal antibody usually showed a speckled pattern in the nuclei (Figure 1), but in some cases staining of the nuclear matrix was prominent.

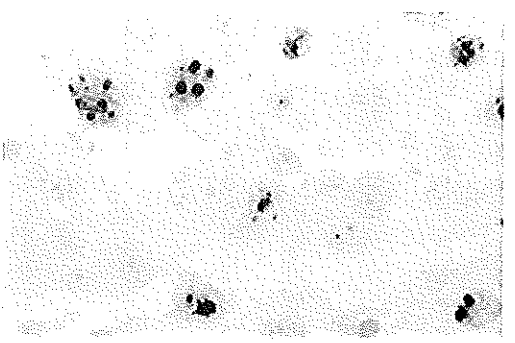

Figure 1: Frozen section of an epithelioid cell-type melanoma incubated with the monoclonal antibody Ki-67 (original magnification, x150). Notice the speckled pattern of Ki-67 immunostaining.

Only rarely, a weak cytoplasmic staining was seen. The percentage of Ki-67 positive cells in all melanomas ranged from 0.16% to 3.80%, and one with a high score of 18.30% (Table 1).

The Ki-67 score of irradiated and non-irradiated eyes differed significantly: A reduction in the Ki-67 score was observed after pre-enucleation irradiation (P= 0.007) (Figure 2). To quantitate this reduction, the natural logarithm of Ki-67 was taken because of its positive skewness and the unequal variance in the irradiated and the nonirradiated group. By comparing the mean logarithm of the Ki-67 score in both groups, it can be estimated that irradiation resulted into approximately a threefold reduction of the Ki-67-defined proliferative fraction. The 95% confidence limits of this reduction are 1.3 and 9.8. Similarly, decreased mitotic rate was found after pre-enucleation irradiation (P= 0.001). A significant correlation was found between Ki-67 score and mitotic rate in the irradiated group, using the Spearman Rank Correlation test (r_S = 0.58; P = 0.002) (Figure 3), but not in the nonirradiated group (r_S = 0.12; P = 0.37) (Figure 3).

Table 1 *Results of Ki-67 score and clinical and pathologic parameters*

Case no	Ki-67 score	Mitotic rate	Cell Type[a]	LTD (mm)[b]	Irr[c]	Loc[d]	Age (yrs)	Sex	Scler[e]	Pigm[f]	Lym[g]
1	0.65	3	M	12	+	P	40	F	4	2	1
2	0.07	1	M	4	+	C	38	M	2	4	1
3	0.24	3	S	13	+	P	56	M	5	2	1
4	0.20	1	S	12	+	P	60	M	2	2	2
5	0.08	0	M	13	+	C	54	M	2	4	1
6	0.11	1	M	12	+	P	59	F	2	4	1
7	0.90	2	M	12	+	P	38	F	3	2	1
8	1.10	1	M	9	+	P	81	M	4	2	1
9	0.07	1	E	9	+	P	58	F	4	2	1
10	0.11	1	S	12	+	E	76	F	1	4	1
11	0.14	0	E	10	+	P	67	M	1	2	1
12	0.96	1	S	11	+	P	50	F	1	3	2
13	0.47	1	S	8	+	P	68	F	1	2	1
14	0.18	1	E	11	+	A	58	F	1	2	3
15	18.3	28	E	18	+	E	69	M	2	2	1
16	3.80	10	E	16	+	P	74	M	4	2	1
17	0.04	2	S	9	+	P	64	F	3	2	1
18	0.02	0	E	12	+	P	74	F	2	4	1
19	0.45	2	E	7	+	P	63	M	2	2	1
20	0.73	5	M	17	-	P	72	F	1	2	2
21	0.32	7	S	10	+	P	57	M	1	2	1
22	0.27	4	M	18	+	A	48	M	3	2	1
23	0.38	2	E	13	+	P	69	F	2	2	1
24	0.16	1	M	12	+	C	71	M	2	2	1
25	3.08	7	M	6	-	P	66	F	1	4	1
26	1.55	8	E	14	-	P	65	M	3	3	3
27	0.52	3	M	6	-	C	66	F	1	2	1
28	1.22	8	S	13	-	P	76	M	2	1	1
29	0.16	3	S	12	-	P	57	F	1	2	1
30	2.60	4	M	11	-	P	77	M	3	1	1
31	0.67	9	S	9	-	P	75	M	5	2	1
32	1.75	3	E	13	-	C	56	M	2	3	3
33	0.83	8	M	18	-	P	64	F	4	3	3

a: *M = mixed; S = spindle; E = epithelioid cell type scored on paraffin embedded tissue.*
b: *largest tumor diameter.*
c: *+ = 8 Gy pre-enucleation irradiation; - = no irradiation.*
d: *location: P = posterior; C = ciliary body; A = anterior; E = equator.*
e: *scleral invasion: 1 = absent; 2 = <25%, 3 = 25-75%; 4 = >75%; 5 = episcleral growth.*
f: *pigmentation: 1 = absent; 2 = <25%; 3 = 25-75%; 4 = >75%.*
g: *lymphocytic infiltration: 1 = absent; 2 = moderate; 3 = marked.*

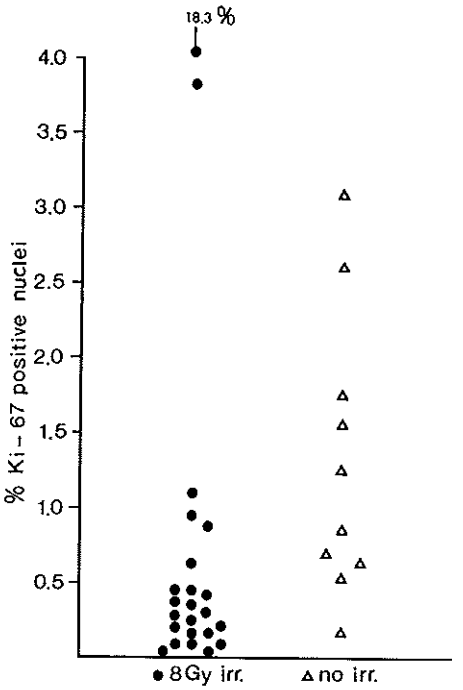

Figure 2. Percentage of Ki-67 positive nuclei in a group of patients, who received 8 Gy pre-enucleation irradiation (●) as compared to a group of patients who did not receive pre-enucleation irradiation (△).

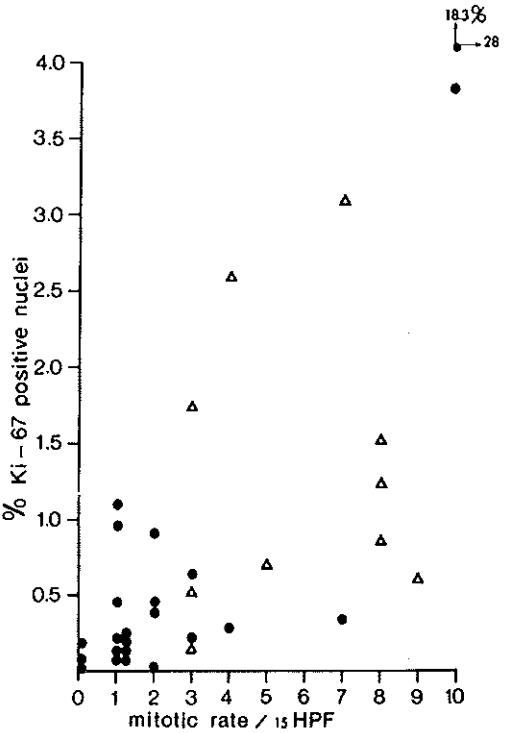

Figure 3. A statistically significant correlation ($P < 0.001$) was found between Ki-67 positivity and mitotic rate. ● : pre-enucleation irradiation (8 Gy). △ : no pre-enucleation irradiation.

No significant correlation could be demonstrated between the Ki-67 score and largest tumor diameter (Figure 4), tumor localization or pigmentation, scleral invasion, and age and sex of the patient. Histologic classification obtained on frozen sections as well as on paraffin-embedded tissue (Figure 5) also did not have a significant correlation with the Ki-67 score. In 12 cases, a discrepancy existed in histologic typing of the tumor between frozen sections and the paraffin-embedded tissue, which can be explained partly by sampling and partly by the unreliability of tumor typing on frozen sections.

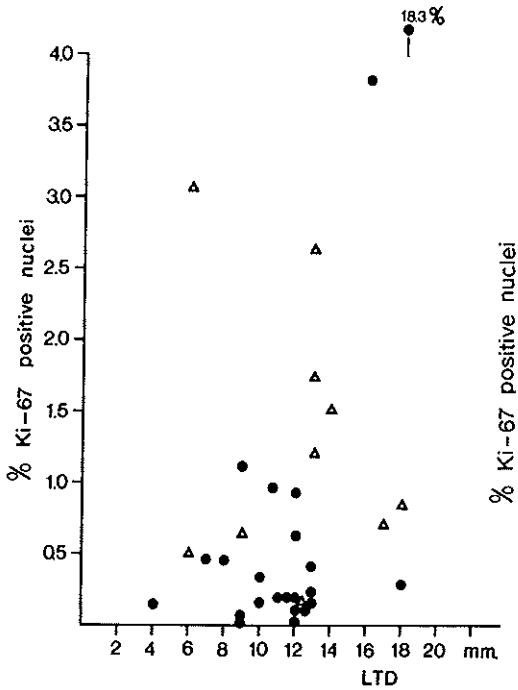

Figure 4. Lack of correlation between largest tumor diameter (LTD) and the % of Ki-67 positive nuclei. ● : pre-enucleation irradiation (8 Gy). △ : no pre-enucleation irradiation.

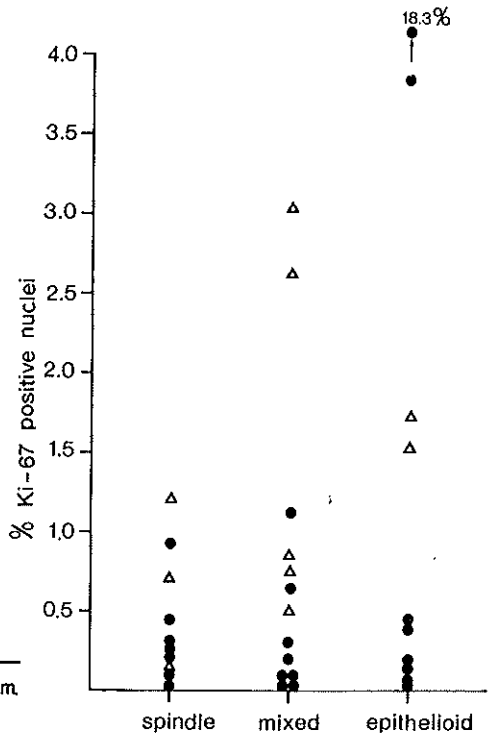

Figure 5. Lack of correlation between Ki-67 positivity and the histological cell type, scored on paraffin embedded tissue. ● : pre-enucleation irradiation (8 Gy). △ : no pre-enucleation irradiation.

To compare our results with bromodeoxyuridine uptake in uveal melanomas, we calculated the mean Ki-67 count in 32 high-power fields in nonirradiated melanomas. The mean count was 128.

The percentage of Ki-67-positive nuclei of the control cutaneous melanomas varied from 1.4% (metastatic cutaneous melanoma) to 4.2%.

Comment

Proliferation rates in uveal melanomas have been retrospectively studied by DNA flow cytometry[14] and by incorporation of bromodeoxyuridine.[15] Both methods have disadvantages: when flow cytometry is used, the proliferative index can only be assessed reliably in diploid tumors. The reported varying incidence of aneuploidy in uveal melanomas (between 4% and 77%[14,16]) may therefore influence the proliferative index. In addition, flow cytometry cannot distinguish between tumor cells alone and tumor cells clumped with nontumor cells. In vivo or in vitro incorporation of the potentially mutagenic DNA bromodeoxyuridine is another frequently used technique to detect cells in the S-phase.[17] A disadvantage of this technique may be that sufficient bromodeoxyuridine incorporation may not occur as a result of poor vascular supply to the tumor or due to limited tissue diffusion.[18] Therefore, we have assessed the growth fraction of ocular melanomas using the monoclonal antibody Ki-67 on frozen sections, which is a simple and reliable method of rapidly evaluating the growth fraction of normal[19-24] and neoplastic human cell populations.[22-32]

Under in vitro conditions, some authors have observed discrepancies between Ki-67 labeling and bromodeoxyuridine incorporation.[33] Nevertheless, in a series of 20 human solid tumors a consistent ratio was found between Ki-67 labeling index (cycling cells/total tumor cells) and bromodeoxyuridine labeling index (S-phase cells/total tumor cells).[6] Comparing the mean Ki-67 count in non-irradiated uveal melanomas in 32 high power fields with the results of a previous study using bromodeoxyuridine uptake in non-irradiated uveal melanomas,[15] the findings are in keeping with this ratio.

In our study the Ki-67 score of nonirradiated uveal melanomas was low (i.e., $<1\%$ to 3.08%) as compared to the Ki-67 score in primary malignant tumors elsewhere in the body, which varies from $<5\%$ to 65%.[8-12,22,24] Furthermore, our results indicate that the Ki-67 score of the nonirradiated choroidal melanomas is generally lower than the Ki-67 score of nonirradiated primary cutaneous melanomas.[34] Recently, a higher Ki-67 score was reported on five choroidal melanomas.[35] These findings are not in keeping with the results using bromodeoxyuridine uptake in choroidal melanomas.[15] Their discrepant findings can be attributed to differences in techniques and selection of patients. Unfortunately, no additional data were provided in their heterogenous series allowing their results for comparison.

The question still remains if Ki-67 immunostaining is relevant for the assessment of

prognosis of patients with ocular melanomas. In our study, there was no correlation between Ki-67 score and conventional prognostic parameters such as histologic classification and largest tumor diameter. These results are similar to the findings for bromodeoxyuridine uptake in uveal melanomas.[15] In this preliminary study, we did not attempt to assess clinical outcome because the follow-up time for the patients from whom we obtained frozen melanoma material was too short. The antigen recognized by Ki-67 does not survive conventional fixation and thus is not suitable for retrospective studies on stored paraffin-embedded tissue specimens. It should be noted, however, that one of the 33 examined patients died only 6 months after enucleation of disseminated choroidal melanoma (epithelioid cell type). Before enucleation, there was no clinical evidence of metastasis. The irradiated choroidal melanoma of this patient had the highest proliferative index (18.3%) in this study, which is comparable with the proliferative index reported for malignant tumors elsewhere in the body. It might be that selection of patients who are at high risk for tumor metastasis could be achieved by looking for a high proliferative index in addition to conventional prognostic parameters as histologic type, tumor size and mitotic rate.

The prognostic significance of the Ki-67 staining remains to be proven. However, conventional counting of mitotic figures yields a poor reflection of the proliferative activity of a given tumor, because only a minor fraction of proliferating cells is in actual mitosis. The mitotic score may be unreliable, due to problems with identification of mitotic figures,[36] variation in cell cycling time or to different handling of the tissue.[37] Nevertheless, in our study a good rank correlation ($r_S = 0.71$; $p < 0.001$) was noted between the Ki-67 score and mitotic rate in the irradiated group, similar to findings in mammary carcinoma,[27] but in contrast to findings in cervical carcinoma.[31]

In patients treated with 20 Gy in 5 fractions over 5 to 7 days before enucleation, pre-enucleation radiotherapy has been shown to decrease the mitotic rate of choroidal melanomas to zero in 15/21 cases.[38] In our study, the mitotic rate was reduced to zero in only 3 of 23 cases.

After more than 60 Gray equivalent (GyE) of helium ion charged-particle therapy, the proliferation rate measured by uptake of bromodeoxyuridine was zero in six of eight patients.[15] A dose of 20 Gy pre-enucleation irradiation gave a 100-fold reduction of bromodeoxyuridine incorporation.[15] The reduction of Ki-67 score in this study was approximately three fold after 8 Gy pre-enucleation dose, which is a small difference with respect to the variability of the KI-67 score in the irradiated group.

The lesser reduction of proliferative activity in our study can be explained by the much smaller radiation dose and the short interval between irradiation and enucleation.

The aim of the low dose irradiation of 8 Gy was to reduce the risk of hematogenous metastases during the enucleation procedure[39] and was not meant to be curative. Whether radiation will only be effective when the number of cells synthesizing DNA is reduced to near zero is a point of discussion.[15]

In conclusion we demonstrated with KI-67 immunostaining a reduction of the proliferative activity of uveal melanomas after pre-enucleation irradiation. The proliferative activity of uveal melanomas was low in comparison to cutaneous melanomas[34] and primary malignant tumors elsewhere in the body. The relevance of Ki-67 immunostaining for the life prognosis of patients with uveal melanomas remains to be established.

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CHAPTER 7

DNA Flow Cytometry in Uveal Melanoma: the Effect of Pre-Enucleation Irradiation

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Summary

For uveal melanoma it has been demonstrated that aneuploidy correlates with worse clinical outcome. However, a striking variation in incidence of aneuploidy is reported for uveal melanomas. Flow cytometry was used to study retrospectively DNA-ploidy of 132 uveal melanomas on paraffin-embedded material. Thirty-five patients received 2x 4 Gy doses of irradiation 24 and 48 hours before enucleation. Correlation between DNA-ploidy and histopathological grading, largest tumour diameter, tumour height, tumour location, scleral invasion, and TNM classification was assessed. Survival analysis methods were used to investigate the predictive value of these variables on clinical outcome.

Of the tumours 37% were aneuploid and 63% were diploid. Intratumour ploidy heterogeneity was minimal (92% concordancy). A strong correlation ($p=0.009$) was found between DNA-ploidy and cell type. No correlation was found between DNA-ploidy and other conventional prognostic parameters.

Irradiated melanomas were significantly more aneuploid than non-irradiated tumours ($p \leq 0.01$). In survival analysis DNA-ploidy and the largest tumour diameter were significant in predicting metastatic outcome ($p \leq 0.03$ and 0.01 respectively); histologic cell type and tumour location were of borderline significance.

Introduction

Ciliary body and choroidal melanomas are the most common primary intraocular malignancy in the adult. The estimated 15-year-survival rate after detection of the tumour is 53%.[1] The metastatic potential of uveal melanomas varies, depending on tumour cell type, largest tumour diameter (LTD), the standard deviation of the nucleolar area and the mean of the largest nucleoli.[2] New treatment modalities, including preoperative irradiation, have not substantially reduced the mortality rate of these tumours.[3]

In a variety of solid tumours, including cutaneous malignant melanoma[4,5] DNA flow cytometry has proved to be a useful and objective prognostic parameter in addition to conventional histopathological classification.[6,7] For uveal melanoma, analysis of recent paraffin-embedded material has shown that aneuploidy correlates with poor prognosis,[8] but on older (> 15 years) archival paraffin-embedded material this was not confirmed.[9] The percentage of aneuploidy in different studies on uveal melanomas varies between 16%[10] and 78%.[11] In the largest study interpretable DNA histograms were obtained in 64 cases, from

which 36% was aneuploid.[8] It has been shown that pre-enucleation irradiation reduces the proliferative activity in uveal melanomas.[12,13] Effects of pre-operative irradiation on tumour-ploidy have not been extensively investigated, however. In an attempt to resolve these inconsistencies, we have studied archival paraffin embedded tissue of 136 uveal melanoma of 13 years old maximum to test the predictive value of aneuploidy on clinical outcome. In the study material we included patients who had received 2x 4 Gy doses of irradiation before enucleation in order to reduce the risk of haematogenous metastases during the enucleation procedure.[14]

The aims of this study were 1) to investigate the incidence of aneuploidy in a large series of cases, 2) to assess intratumour heterogeneity, 3) to investigate the effect of pre-enucleation irradiation on DNA-ploidy, and 4) to investigate the effect of DNA-ploidy on clinical outcome. Estimation of S-phase fractions was not performed, because of unreliability of this method on paraffin embedded material.[15]

Materials and Methods

From 1976 to 1989, 98 paraffin blocks and 5 frozen specimens from patients with choroidal and ciliary body melanomas were collected from the department of pathology, Erasmus University Rotterdam. Thirty-eight patients received 2x 4 Gy doses of irradiation before enucleation. Forty-three paraffin blocks from non-irradiated tumours from the same period were selected from the department of pathology, University of Nijmegen, totalling 146 cases (Table 1). Until 1993 adequate follow-up of 97 patients could be obtained by contacting the local ophthalmologist or the general physician. Follow-up data were requested and verified. Thirty-four patients died from tumour related causes, 50 patients were still alive and 13 patients died of other causes (Table 2).

Three 50 μm sections were cut from the paraffin blocks. Additional 7 μm sections were obtained from each side of the experimental material and stained with haematoxylin and eosin for histopathological evaluation. Eighty blocks contained more than 75% tumour tissue and 66 blocks contained normal ocular tissue as well. Seven normal eyes without tumour were also examined. Of the 50 μm sections, nuclear suspensions were processed by the method of Hedley et al.[16] The paraffin was dissolved with two washes of xylene. All sections were rehydrated with two 10 minute washes in 100% alcohol, one wash with 96% alcohol, and two washes with 70% and 50% alcohol. The samples were rinsed with distilled water, and centrifuged for 10 minutes at 800 *g*. The centrifuged tissue was

suspended in a test tube containing 0.5% pepsin in 0.9% sodium chloride (pH 1.9 plus 0.02% azide) and incubated for 1 hour at 37°C with repeated vortexing, centrifuged at 800 *g*. and subsequently the cells were resuspended in Hank's balanced salt solution containing ethidium-bromide (50 μg/ml). The samples were filtered through a 40 μm. nylon mesh filter. The stained samples were measured on a FACS Scan (Becton Dickinson, Sunnyvale, CA). For each histogram 10^6 nuclei were analysed. In these paraffin-embedded tissues, artefactual low-staining debris and cell clumps tend to be present. Confounding effects of these signals were eliminated by setting a gate.

Of 15 cases, a second paraffin block from a different area of the neoplasm was obtained to investigate intratumour heterogeneity.

The five freshly obtained tumours had all been irradiated preoperatively. These samples were prepared and stained according to the method of Vindeløv et al.[17]

Data Analysis

The DNA-ploidy of the tumour sample was estimated by DNA index, which was calculated as the ratio between the median channel numbers of the first and the subsequent peaks in the sample. Tumour samples were accepted as diploid where there was a single G0/G1 peak. Samples with a coefficient of variation (CV) of the single diploid peak of more than 9% were excluded. The CV was calculated as the full width of the G0/G1 peak at half maximum divided by the mean channel number. In cases with multiple peaks, the population with the lowest DNA content was assumed to represent the diploid population. In the samples from normal eyes the percentage G2M ranged from 3% to 6%. Therefore we defined a histogram as tetraploid if the second peak had a DNA index between 1.9 and 2.1 and the fraction contained more than 8% of the nuclei measured. Samples with a DNA index of the second peak of more than 2.1 or less than 1.9 or with a first peak with a shoulder were defined as DNA aneuploid.

Histopathological Grading

The tumours were classified as spindle cell, epithelioid cell, or mixed cell type according to modified Callender's classification. Five other variables were measured: largest tumour diameter (LTD, <7 mm, 7-10 mm, 10-15 mm, >15mm); tumour height (<2 mm, 2-3 mm, 3-5 mm, >5 mm); tumour localisation (ciliary body, equator, posterior, diffuse) and scleral invasion (none, less than 50%, 75%, episcleral growth). In addition the tumours were classified according to the TNM system (WHO).

Statistical Analysis

Cross tabulation together with the X^2 test were used to compare DNA-ploidy with the 6 above described variables. For ordinal variables, the Cochran-Mantel-Haenszel trend version of the X^2, as implemented in SPSS, was performed. Distribution of (tumour related) survival is described by Kaplan-Meier curves, which were compared with the log rank test. Where appropriate a trend version of this test was performed.

Results

Seventy-two patients were male, and 74 were female. The mean age was 61.6 year. A total of 146 tumour samples were analysed. Fourteen cases were excluded, because of a high CV- three of whom had received pre-enucleation irradiation (Table 1); in 12 of these follow-up was known (Table 2). On the remaining 132 cases possible correlations between the various parameters were analysed. Influence of histological cell type, LTD, tumour prominence, tumour localisation, scleral invasion, and TNM classification on survival was studied in 97 patients with adequate follow-up (Table 2), whereas the influence of DNA-ploidy on survival was studied in 85 patients with adequate follow-up (Table 2).

The total mean follow-up was 62 months. DNA-ploidy, and LTD were significant in predicting metastatic potential ($p \leq 0.03$, and 0.01 respectively) (Figs 1 and 2); histological cell type and tumour location were of borderline significance ($p \leq 0.05$).

Table 1 *Patient Selection*

	Total No.	Excluded No.*	Remaining No.
2x 4 Gy. irr.	38	3	35
no irradiation	108	11	97
total	146	14	132

* *Excluded because of a high coefficient of variation (CV).*

Table 2 *Number of Patients with Known Follow-up*

	Total No.	Excluded No.*	Remaining No.
Tumour related death	34	5	29
Alive	50	5	45
Non-tumour rel. death	13	2	11
No follow-up	49	2	47
total	146	14	132

* *Excluded because of a high coefficient of variation (CV).*

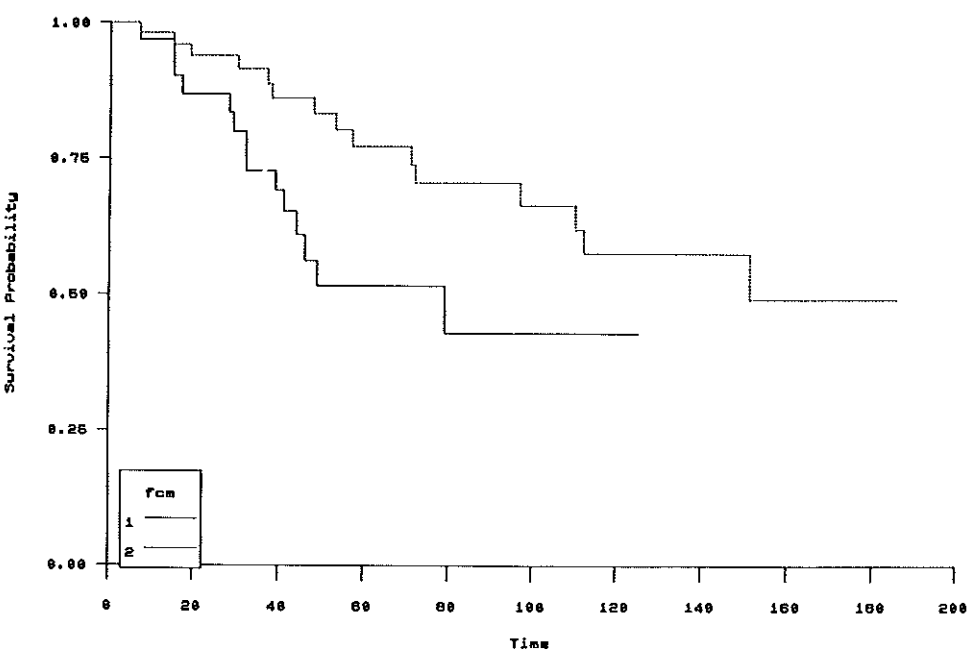

Figure 1: Kaplan-Meier survival curves (tumour related death) as a function of DNA-ploidy in a series of 85 patients. 1: aneuploid melanomas; 2: diploid melanomas; Time in months after enucleation.

Thirty-seven percent of the samples were aneuploid, including 17% of the tumours which were tetraploid; 63% were diploid (Table 3). A strong association ($p \leq 0.0009$) was found between DNA-ploidy and cell type (Table 3). This association remained significant after correction for LTD ($p \leq 0.009$) and TNM classification ($p \leq 0.008$) by stratification.

No correlation was found between DNA-ploidy and LTD, tumour height, scleral invasion, tumour location, and TNM classification.

A significant association was found between aneuploidy and pre-enucleation irradiation ($p \leq 0.01$) (Table 3).

Normal eye tissue was diploid, as assessed on paraffin blocks of seven eyes without abnormalities. Of 12 patients two tumour samples were analysed: in 11 of these (92%) only one DNA peak was found. The five fresh tumours were all aneuploid, including one tumour of the epithelioid cell type.

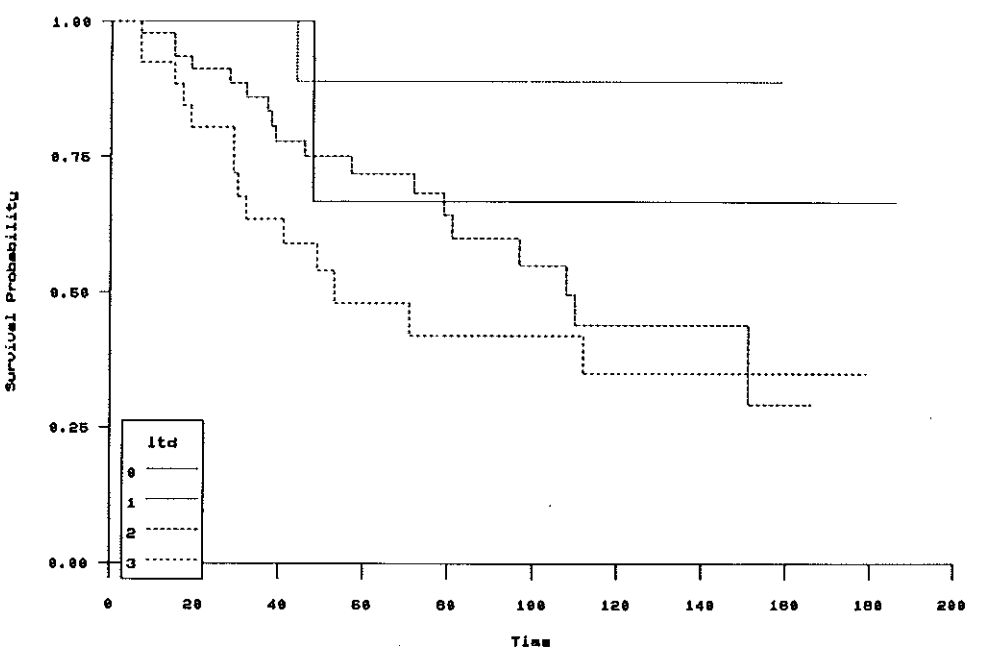

Figure 2: Kaplan-Meier survival curves as a function of Largest Tumour Diameter (LTD) in a series of 97 patients. 0: LTD:< 7 mm; 1: LTD: 7-10 mm; 2: LTD: 10-15 mm; 3: LTD: > 15 mm. Time in months after enucleation.

Table 3 *Crosstabulation DNA-ploidy and Clinicopathologic Features*

	Aneuploid	Diploid	Total No.
Histological type			
Epithelioid	15 (60%)	10 (40%)	25 (100%)
	(31%)	(12%)	(19%)
Mixed	24 (42%)	33 (58%)	57 (100%)
	(50%)	(39%)	(43%)
Spindle	9 (18%)	41 (82%)	50 (100%)
	(19%)	(49%)	(38%)
Total	48 (37%)	84 (63%)	132 (100%)
	(100%)	(100%)	(100%)
Pre-enucleated Irradiation			
no irradiation	29 (30%)	68 (70%)	97 (100%)
	(60%)	(81%)	(73%)
2x 4 Gy	19 (54%)	16 (46%)	35 (100%)
irradiation	(40%)	(19%)	(27%)
Total	48 (37%)	84 (63%)	132 (100%)
	(100%)	(100%)	(100%)

Discussion

There is increasing evidence for a variety of neoplasms that DNA aneuploidy may correlate with poor prognosis.[18] In primary cutaneous melanomas DNA aneuploidy has been shown to correlate with tumour thickness, incidence of recurrence, and survival.[5] Studies in uveal melanoma demonstrated that an elevated DNA index (> 1.4) is strongly correlated with higher tumour related mortality.[8] We found aneuploidy in 37% of the cases, which is consistent with the findings of Meecham & Char.[8] The striking variation in incidence of

aneuploidy in earlier studies can partly be explained by the relatively small number of cases studied[10,11] and the use of fresh tissue.[10] Another explanation may be the differences in the applied techniques, like assessing DNA content on older archival paraffin embedded specimens. In older material increased background noise from fragmented nuclei and cellular debris is important, and as sample age increases, such problems for DNA content seem to increase.[19] Up to a period of 10 years the age of the block does not appear to influence the CV,[20] because comparison of DNA-ploidy of fresh tissue samples with formalin-fixed paraffin embedded specimens in solid tumours showed a concordancy of 87%.[21] None the less, in the latter study near-diploid aneuploid peaks observed in histograms from fresh tissues were sometimes not apparent in histograms from paraffin-embedded tissues, because of the higher CV for the latter samples. Another problem in paraffin-embedded specimens is that, if both diploid and near-diploid aneuploid peaks are present, it is difficult to determine which peak is diploid. To obviate this particular problem in 66 cases we measured a mixture of nuclei from a paraffin block of normal tissue and tumour tissue from the same specimen as recommended by Schutte et al.[22]

A further explanation for the variable percentage of aneuploid cases reported is tumour heterogeneity. In a small tumour sample an aneuploid subpopulation of cells might remain undetected. However, in the cases in which we could study two blocks, 92% was concordant. We found the predictive value of aneuploidy for survival to be significant ($p \leq 0.03$), contrasting with a study on older formalin-fixed paraffin-embedded specimens.[9] Our findings indicate that specimens can be used for flow cytometry at least up to 10 years after preparation.

Although simplification of the original Callender classification has improved histologic correlation with malignancy, interobserver error can be large.[23] Subsequently, a more quantitative system was developed to estimate subjectively the percentage of epithelioid cells in each tumour,[24] however still relying on subjective judgement. Therefore methods have been developed to measure more objective features. One of these methods is determining DNA ploidy. We have demonstrated that archival paraffin-embedded material can be used for flow cytometry analysis to predict clinical outcome. We found that aneuploidy in uveal melanomas strongly correlates with epithelioid cell type, none the less, aneuploidy appears to have a better predictive value for prognosis than subjective histopathological classification. In our study we found that cell type and tumour location were of borderline significance in predicting clinical outcome. This is in contrast to earlier reports on prognostic factors in uveal melanoma,[25] but in agreement with recent findings of

Folberg et al.[26]

Interestingly, we found irradiated melanomas to be significantly more often aneuploid than non-irradiated melanomas. Radiation induces mitotic delay and both numerical and structural chromosome aberrations.[27] It is conceivable that during cell cycle progression alterations of chromatin condensation and DNA fluorochrome labelling lead to DNA damage which, in flow cytometry, may give rise to the appearance of pseudo-aneuploid cell populations.[28] Numerical chromosomal aberrations may give rise to near diploid or near tetraploid histograms. However, the decline in radiation induced aberrations in the initial 24 hours is rapid, presumably as a result of DNA repair.[29] In vitro studies on a K-1735 melanoma cell line exposed to 7 Gy. X-irradiation revealed that increase in karyotype diversity generated by radiation, when nonlethal, may accelerate tumour progression.[30] Unfortunately no paraffin tissue was left to study the relationship between DNA aneuploidy and chromosomal aberrations in irradiated melanomas.

We demonstrated a strong correlation ($p \leq 0.009$) between aneuploidy and epithelioid cell type. Other studies indicated a similar correlation, but were too small to draw statistical conclusions.[10,31] Aneuploidy may reflect a genetically more unstable population with an enhanced ability to metastasize.

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CHAPTER 8

An Immunohistochemical and Prognostic Analysis of Apoptosis and Proliferation in Uveal Melanoma

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Summary

Neoplasia can be defined as deregulated tissue homeostasis due to an imbalance between proliferation and apoptosis. Many genes are involved in the maintenance of tissue homeostasis e.g. the c-myc oncoprotein, which is an important regulator of cell proliferation and Bcl-2 protein, which is involved in the regulation of apoptosis. We studied retrospectively indices of proliferation, such as mitotic count and the Mib-1 index, on 51 uveal melanomas and compared their prognostic significance with established indicators of prognosis such as cell type and tumor size. Along the same line we investigated the expression of the regulating proteins c-myc and Bcl-2. Of all parameters tested, the largest tumor diameter (LTD) and mitotic count were most strongly associated with tumor-related death ($p < 0.001$ and $p = 0.005$ respectively). In addition, cell type, the presence of epithelioid cells, the Mib-1 index, and the percentage of cytoplasmic c-myc positive cells were significant predictive factors. Multivariate analysis showed that the Mib-1 index, LTD and the percentage of cytoplasmic c-myc positive cells were independent prognostic parameters. Bcl-2 expression did not correlate with clinical outcome. The Mib-1 index correlated with the presence of epithelioid cells ($p < 0.03$), the presence of apoptotic bodies ($p < 0.001$) and c-myc. A strong inverse relationship was found between (nuclear and cytoplasmic) c-myc and Bcl-2 ($p < 0.00004$ and $p < 0.006$ respectively), suggesting that Bcl-2 cooperates with c-myc to immortalize uveal melanoma cells.

Introduction

The maintenance of homeostasis in normal tissue can be viewed as a tightly regulated balance between cell production and cell death.[1] Neoplasia can arise when tissue homeostasis is deregulated. Most of the knowledge concerning oncogenic events has concentrated on mechanisms of increased cell growth. However, decreased cell death also would result in an expansion of the cell mass.[1] Cells can die either by necrosis (inactively) or by apoptosis (actively). Individual cell disintegration is a constant finding in malignant neoplastic tissue and these dying or dead cells, morphologically characterized by volume contraction and nuclear condensation have been called apoptotic.[2] Monoclonal antibodies (MAbs) against proteins involved in the regulation of cell proliferation and death can be used to visualize the dynamics of tissue homeostasis. The Bcl-2 protein blocks apoptosis and thus prolongs cell survival. In human fetal tissues Bcl-2 appears to be involved in

tissue homeostasis as well as morphogenesis.[3] Only few reports concerning Bcl-2 expression in non-hematopoietic malignancy have been published,[4-7] including cutaneous melanoma.[8] The c-myc protein is involved in the control of cell proliferation, but is also a potent inducer of apoptosis.[9] C-myc expression is frequently deregulated in neoplasms and is often implicated in their genesis.[10] The c-myc gene is located on chromosome 8q24.1; chromosomal abnormalities involving chromosome 8q have been specifically associated with uveal melanoma.[11] It has been found that staining for c-myc protein correlates with proliferative index in diploid uveal melanomas, supporting the hypothesis that c-myc protein is involved in cellular proliferation.[12] Proliferative indices may provide information independent of other histological and clinical prognostic variables.[13] The MAb Mib-1 recognizes the Ki-67 antigen, which is expressed by proliferating cells and can be used on formaldehyde fixed paraffin sections.[14]

The purpose of this study was to determine whether or not the expression of c-myc, Mib-1, Bcl-2 and the mitotic rate in uveal melanomas have independent prognostic significance in comparison to cell type and tumor size. Furthermore we investigated the associations between the proteins involved in the regulation of cell proliferation and death.

Materials and Methods

Histologic Specimens

In order to correlate immunohistochemical findings with prognosis, a retrospective analysis of 51 formalin fixed paraffin-embedded uveal melanomas was undertaken. From 1973 to 1987 consecutive cases were entered in the study on the basis of availability of adequate histologic material. Follow-up data were obtained by contacting the local ophthalmologist and/or the general practitioner, and these data were reviewed in order to define tumor-related death or death due to other causes.

In order to test the antibody specificity we used frozen tissue from one of the patients included in this series and a cell line (OMM-1), obtained from metastatic uveal melanoma tissue.[15] A colon carcinoma and a breast carcinoma served as a control for c-myc.

Paraffin sections were cut at 5 to 6 μm and stained with hematoxylin-eosin. In these sections we determined the following parameters: largest tumor diameter (LTD) ($\leq$10 mm, 10-15 mm, $>$15 mm), cell type, mitotic rate and the presence of apoptotic bodies. The tumors were histologically classified in two groups: 1) according to cell type, using the three categories of the modified Callender classification (spindle cell, mixed cell and

epithelioid cell type)[16] and 2) according to the presence or absence of any epithelioid cells (spindle cell melanoma versus a combination of mixed cell type and epithelioid tumors).[17] Mitoses were counted in 15 high power fields (HPF) with a total magnification of x400, using an eye piece grid. This was repeated three times and the number of mitoses was averaged. Apoptotic bodies were recognized by volume contraction and nuclear condensation of tumor cells.[5] Using light microscopy, uncertainties in defining apoptotic bodies remain, therefore we did not use an index for apoptotic bodies,[5] but scored for the presence or absence of apoptosis.

Areas with tissue necrosis were excluded from the counting.

Immunohistochemistry

Formalin fixed and paraffin embedded 5 μm sections were mounted on aminopropyltriethoxysilane (APES, Sigma, St Louis, USA) coated glass slides and dried overnight at 37°C. After deparaffinizing and rehydrating, the slides were placed in 0.01 M citrate buffer and antigen retrieval was performed by microwave irradiation (Bio-Rad) for 2x 5 min. The slides were pre-incubated with normal goat serum in a dilution of 1:10 for 15 minutes.

The following specific antibodies were used:

1) The MAb raised against the C-terminal peptide (9E10) (amino acids 408 through 439) of the human c-myc protein (Oncogene Science Inc. New York, USA) was used in a dilution of 1:1600 and incubated overnight. The slides were incubated for 30 minutes at room temperature (RT) with biotinylated multilink immunoglobulin (Ig, Biogenex) in a dilution of 1:75, in phosphate-buffered saline (PBS) with 5% BSA. After washing in PBS/Tween 0.5% the slides were incubated with the streptavidin-biotin-peroxidase complex (Biogenex) in a dilution of 1:50. The alkaline phosphatase anti-alkaline phosphatase (APAAP) technique was used as detection system with fast red as chromogen. As specificity control, sections were incubated with antibody preincubated overnight with 10 μg/ml excess of the peptide (Oncogene Science, New York, USA). 2) The MAb MIB-1, reacting with the proliferation associated antigen Ki-67 (Dianova-Immunotech, Hamburg, Germany) was used in a dilution of 1:200, in an overnight incubation protocol at 4°C.

3) The MAb specific for Bcl-2 oncoprotein (clone 124) was obtained from Dakopatts (Glostrup, Denmark) and used in a dilution of 1:60. Slides were incubated for 60 minutes at RT.

After incubation with MAbs Mib-1 and Bcl-2, the slides were incubated for 30 minutes at

RT with biotinylated goat-anti mouse Ig (Dakopatts) in a dilution of 1:400, in PBS with 2% human serum and normal goat serum. After washing in PBS, the slides were incubated with the streptavidin-biotin-peroxidase complex (Dakopatts) in a dilution of 1:200. The peroxidase was visualized using hydrogen peroxide in N-N-dimethylformamide with 3 amino-9 ethylcarbazole dimethylformamide as chromogenic substrate.
As a negative control, specimens were stained following the same incubation protocol without use of the primary MAbs. All sections were counterstained with Mayer's hematoxylin and mounted with glycerin/gelatin.
As positive control for c-myc, Bcl-2 and Mib-1 sections of a breast carcinoma, normal thymus and adenocarcinoma of the prostate, respectively were used. In addition cytospin preparations of OMM-1 cells were used as positive control for Bcl-2 and c-myc.

Assessment of Results
Immunohistochemical results were evaluated without access to the follow-up data. The Mib-1 score was determined as the percentage of Mib-1 positive cells relative to the total number of cells per HPF. Cell nuclei were considered to be positive if there was any nuclear staining present, regardless of the intensity and distribution within the nucleus. C-myc and Bcl-2 scores were semi-quantitatively determined as percentage of cytoplasmic or positive cells 0, 1-25%, 25-50%, 50-75%, 75-100%. Nuclear staining of c-myc was scored similarly, with an additional score for focal (<5%) staining.

Western Blotting
The specificity of the c-myc and Bcl-2 monoclonal antibodies for use in immunohistochemistry was determined by Western blotting of a total protein extract from frozen uveal melanoma tissue and OMM-1 cultured cells. Frozen tissue from a colon-and a breast-carcinoma served as control for c-myc. The frozen tissue was homogenized in a buffer containing a mix of proteinase inhibitors. OMM-1 cells were harvested using a cell scraper, sonicated, and freeze/thawed. The homogenate was boiled in denaturation buffer (0,1% dithiothreitol; 1% sodium dodecylsulphate; 10% sucrose; Tris/HCL) for 5 minutes. The proteins were loaded on a SDS/Polyacrylamide-gel (12%). The gel was blotted overnight (0.2 A; 33 V, 4°C) on Immobilon P (Millipore) and incubated at RT with 2% BSA/0,1% Tween-20/PBS and subsequently with 1% goat serum in 0,1% Tween-20/PBS for 20 minutes. The dilution used for Bcl-2 and C-myc was 1:3000 and 1:100, respectively. Incubation was performed at RT for 2 hours. Peroxidase-conjugated rabbit-

anti-mouse Ig (DAKO) was used as a second antibody in a dilution of 1:10.000. The peroxidase was visualized by the enhanced chemiluminescence method (Amersham). Between each incubation step the blots were rinsed five times with PBS/0,1% Tween-20 for 1/2 hour.

Statistical Analysis

Spearman's correlation coefficient was used to determine the associations between the variables mitotic rate, presence of apoptotic bodies, Mib-1 score, the percentage of c-myc (cytoplasmic or nuclear) and Bcl-2 positive cells, LTD, cell type and presence of epithelioid cells.

The Kruskal-Wallis test was used to determine the relation between the variables cell type, Mib-1 score and the percentage of positive c-myc (cytoplasmic or nuclear) and Bcl-2 positive cells. The Mann-Whitney-U-Wilcoxon test was used to evaluate the association between the presence of epithelioid cells, the Mib-1 score and the percentage of c-myc (cytoplasmic or nuclear) and Bcl-2 positive cells.

The logranktest and Cox proportional hazards analysis were used as univariate and multivariate regression analysis to assess the influence of different potential prognostic factors on survival. A p value <0.05 was considered significant.

Results

Clinicopathological Parameters

The mean age at diagnosis was 59.8 years. Thirty-two patients were male, 19 female. Twenty-three patients died of tumor related death, 9 died of other causes, 13 were still alive, and 6 were lost to follow-up. The total mean follow-up was 83.9 months.

Twenty tumors were classified as spindle cell type, 19 as mixed cell type and 12 as epithelioid cell type: in 32 of 51 tumors epithelioid cells were present. Five tumors were small (< 10 mm), 24 were 10-15 mm, and 22 were large (> 15 mm).

The mitotic rate was low (<2 mitoses per 15 HPF) in 39 of 51 tumors. In 12 tumors a mitotic rate of ≥2 per 15 HPF was noted, 6 of these patients died of tumor-related death.

Apoptotic bodies were relatively abundant in one tumor (Figure 1a) and could only sporadically be observed in 10 other tumors.

Antibody Specificity

OMM-1 cells and the frozen tissue displayed a weak reactivity for c-myc and strong reactivity for Bcl-2. The anti c-myc antibody recognized a protein in OMM-1 cells (Figure 2), uveal melanoma cells, and in the carcinomas of approximately 40 kD. In the carcinomas, in addition a specific doublet was noted at 65 kD. In the slides, which were pre-incubated with the peptide antigen, the specific reaction with the c-myc antibody was eliminated.

The anti-Bcl-2 antibody bound a protein in OMM-1 cells with an apparent molecular weight of 25 kD (Figure 2), which is in agreement with the described molecular weight of Bcl-2 in other tumors.[4]

Immunohistochemistry

In 15 tumors the Mib-1 score was > 1,8% (Figure 1b), eight patients in this group died of tumor-related death.

In one tumor c-myc staining could not be reliably assessed. In 16 (33%) both nuclear and cytoplasmic staining was noted (Figure 1c); in 40 (78%) only cytoplasmic staining. The distribution of the scores is reflected in Figure 3. As internal positive control in the same sections non-tumor ocular tissue staining was noted in the photoreceptor inner segments of the retina.

In 49 melanomas cytoplasmic bcl-2 staining was found (Figure 1d), two were negative. The distribution of the scores is reflected in Figure 3. In non-tumor ocular tissue staining of Bcl-2 was noted as an internal positive control in normal choroidal melanocytes, the retinal pigment epithelium, the non-pigmented epithelium of the ciliary body, tumor infiltrating lymphocytes, in the Müller cells, the plexiform layers of the retina and the glial cells of the optic nerve.

In the one tumor with abundant apoptotic bodies Bcl-2 expression was low, whereas c-myc (nuclear and cytoplasmic) expression and the proliferative activity were high (19 mitoses per 15 HPF, Mib-1 score: 2.68%).

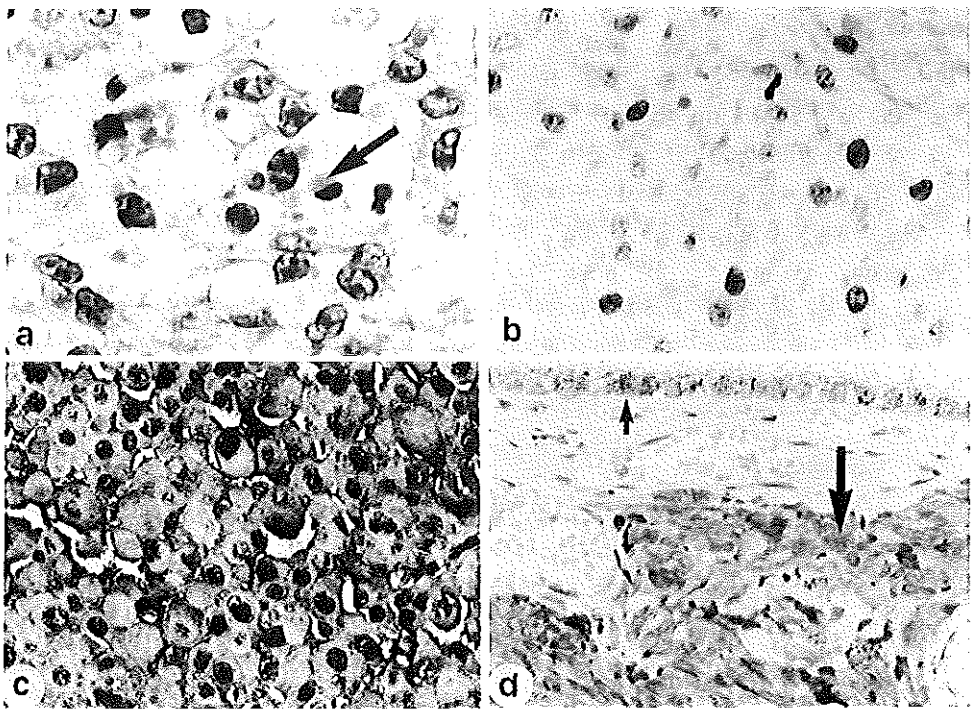

Figure 1 a) Apoptotic body (arrow) in uveal melanoma, characterized by nuclear condensation and volume contraction. (hematoxylin-eosin, magnification x880). b) Immunohistochemistry of a section incubated with Mib-1 (magnification x361). Note the speckled nuclear staining pattern. c) Immunohistochemistry of a section incubated with c-myc with strong nuclear staining (magnification x361). d) Immunohistochemistry of a section incubated with Bcl-2 with positive staining of uveal melanoma (long arrow). The retinal pigment epithelium is indicated by the short arrow (magnification x361).

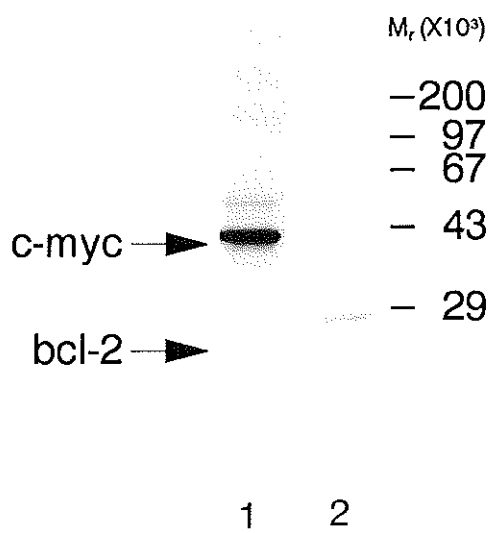

Figure 2: Western blot analysis from cell line OMM-1 with c-myc (lane 1) and Bcl-2 (lane 2).

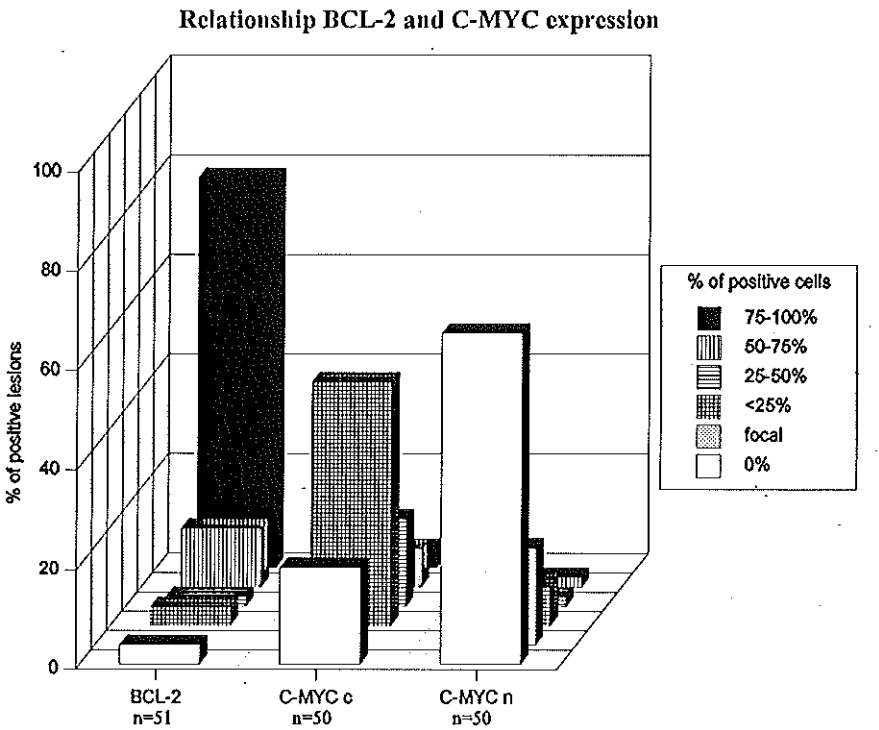

Figure 3: Percentage of nuclear and cytoplasmic c-myc positive cells and Bcl-2 positive cells, expressed as a percentage of total number of lesions. C-myc c: cytoplasmic staining; c-myc n:: nuclear staining

Statistical Analysis

The Mib-1 score correlated significantly with mitotic rate ($p < 0.003$), the presence of apoptotic bodies ($p < 0.001$) and the presence of epithelioid cells ($p < 0.037$). The mitotic rate correlated significantly with cell type ($p < 0.04$).

A strong inverse relationship was found between nuclear/ cytoplasmic c-myc positive cells and Bcl-2 expression ($p < 0.00004$/ $p < 0.006$ respectively) (Figure 3). The percentage of c-myc cytoplasmic positive cells correlated with the Mib-1 index ($p < 0.01$), however, nuclear c-myc protein staining did correlate significantly with Bcl-2 staining ($p < 0.09$).

The logrank test revealed a significant correlation of survival with cell type ($p < 0.05$), the presence of epithelioid cells ($p < 0.05$), LTD ($p < 0.001$), mitotic rate ($p < 0.005$), the Mib-1 score ($p < 0.04$), and the % of c-myc cytoplasmic positive cells ($p < 0.05$), but not with Bcl-2 staining.

In the multivariate analysis with the Cox proportional hazard model, the correlation of LTD, the Mib-1 index and % of c-myc positive cells with survival remained significant after correcting for the influence of other investigated parameters.

Discussion

We have demonstrated that the percentage of c-myc cytoplasmic positive cells is significantly correlated with the Mib-1 index, which is in line with the involvement of c-myc in maintaining cell proliferation. In agreement with the findings on PC-10 (proliferating cell nuclear antigen)[18] staining but in contrast to earlier findings with Ki-67 defined proliferative activity,[19] we found the mitotic rate and the Mib-1 score to be correlated. The latter discrepancy is probably due to a difference in the methods used to define the number of tumor cells per HPF, which affects the Mib-1 score. We also showed specific c-myc staining in uveal melanoma cells. The molecular weight for c-myc we found in immunoblots of uveal melanoma is less than the molecular weight described for c-myc in other tumors.[20] However, it has been demonstrated that two proteins of 32 kD and 58kD detected in extracts of human cells are antigenically related to the synthetic peptides, to which the antibody was raised.[21] Furthermore it has been shown that clone 9E10 reacts by immunoprecipitation and immunoblotting with human c-myc encoded 67 kD and its cleavage products.[22] The use of a neutralized antibody excluded nonspecific effects.

Staining for c-myc protein was found in both the nucleus and the cytoplasm. This was earlier reported by Royds et al.[12] but in a higher percentage of lesions than in our series. However, all their melanomas were of the mixed and epithelioid cell type, and classified as large tumors (>15 mm), whereas our study contained 39% spindle cell melanomas and 57% small and medium sized tumors. The cytoplasmic localization of c-myc was unexpected. The c-myc gene encodes two nuclear phosphoproteins.[10,20] It has been suggested that newly-synthesized myc protein is retained in the polyribosomes. Upon activation of this system, c-myc would be released to the nucleus, where it has been shown to bind both to specific and relatively non-specific DNA sequences, perhaps influencing DNA replication.[23] Aberrant expression of c-myc protein may both result from and contribute to deregulation of cell proliferation, with altered nuclear import and processing of c-myc leading to cytoplasmic accumulation of the protein.

Furthermore, we found a strong inverse relationship between c-myc (nuclear as well as cytoplasmic) and Bcl-2 immunoreactivity. Expression of c-myc is strongly implicated in the

control of cell growth and proliferation: c-myc and other cell cycle related genes play an important role in the G0-G1/S phase transition.[24] In addition c-myc recently proved to be a potent inducer of apoptosis when expressed in the absence of serum or growth factors.[9,25] These opposing roles of c-myc in cell growth and cell death are modified by Bcl-2: it has been demonstrated that Bcl-2 prevents apoptotic cell death induced by c-myc, providing a mechanism whereby cells can express c-myc without undergoing apoptosis.[26-29] We found apoptosis not to be a prominent feature in uveal melanomas.

Fibroblasts which express c-myc do not undergo growth arrest in low serum concentrations as do wild-type fibroblasts, but undergo apoptosis.[25] It has been shown that expression of Bcl-2 protein specifically abrogates c-myc-induced apoptosis without affecting c-myc mitogenic function.[30] This may explain the synergism between c-myc and Bcl-2 in certain tumors.[27]

The most important findings in this study are that in addition to LTD, the Mib-1 index and the percentage of cytoplasmic c-myc positive cells are useful independent prognostic parameters for ciliary body and choroidal melanomas. This has been suggested in earlier studies of Ki-67 on frozen sections of uveal melanomas,[19] and in a recent study of PC-10[18] and c-myc on wax embedded uveal melanomas.[12] Bcl-2 did not appear to be of prognostic significance.

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CHAPTER 9

Components of the Plasminogen Activation System in Uveal Melanoma: a Clinicopathological Study

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Summary

In tumor development, proteases like plasminogen activators (PAs) play a role in degradation of the extracellular matrix and other tissue barriers. Recently, we demonstrated that plasminogen activators, their inhibitors, and urokinase receptor emerge in late stages of cutaneous melanocytic tumor progression. In this study we investigated the expression and distribution of the various components of the PA system and the presence of PA enzyme activity in 45 freshly frozen primary uveal melanomas with known follow-up (14 spindle and 31 non-spindle-type) and in metastases (n=5). Tissue-type PA (t-PA) was found in endothelium of blood vessels and in tumor cells in almost all lesions. t-PA was markedly present at the invasive front (towards the sclera and Bruch's membrane), but no correlation with tumor related death could be established. Urokinase PA (u-PA) was expressed focally, only by 5 non-spindle cell melanomas and in all metastases. u-PA expression correlated with occurrence of metastasis. u-PA receptor (u-PAR) was present in one third of all lesions examined. Plasminogen activator inhibitors (PAI-1 and PAI-2) were only found focally in approximately ten percent of the lesions. In all metastases staining of t-PA, u-PA and PAI was observed.

We conclude that in uveal melanoma, u-PA expression may be associated with metastatic disease and accordingly with poor prognosis. Further research on a large group of tumors with known follow-up is needed to establish whether u-PA positivity is of additional prognostic value in uveal melanoma.

Introduction

To migrate from the primary tumor into blood vessels and to home and grow at a distant site in the body, tumor cells need an extensive repertoire of proteins (1). For migration through and breakdown of tissue barriers, proteases are required (2). An important proteolytic system involved in tumor progression comprises the proteins of the plasminogen activator system (3,4). The two known plasminogen activators (PAs) are tissue-type plasminogen activator (t-PA) and urokinase type plasminogen activator (u-PA). PA activity can be inhibited by specific PA inhibitors, PAI-1 and PAI-2 (5). u-PA activity can be focused at the cell surface by binding to its receptor (u-PAR). Also, u-PA activity can be enhanced by binding to the receptor, as the conversion of the inactive pro-u-PA to active u-PA is facilitated on the cell surface (6,7). Furthermore, by rapid internalization of u-PAR

saturated with u-PA:PAI-1 and recycling of u-PAR, the availability of u-PAR for u-PA binding is ensured (8-10). The saturation of the u-PAR is either autocrine or paracrine (11-13).

The actual involvement of PAs in tumor cell migration and metastasis formation has been shown in several model systems (14-16). Elevated levels of u-PA, u-PAR and PAI in human malignant tumor tissue relative to the benign precursor lesion or normal tissue have been reported for tumors of diverse origin (17-22). Furthermore, u-PA content has shown to be a valuable prognostic marker for mammary carcinoma (19,23). The role of t-PA in tumor progression however, is more ambiguous. Lower levels of t-PA in tumors compared to benign lesions have been reported for tumors of the mammary gland (24) and the ovary (21). Regarding cutaneous melanoma, some reports indicate a high expression of t-PA in melanoma cell lines (16,25,26) and cutaneous melanoma lesions (25,26).

We recently demonstrated both in a nude mouse model (16) and in fresh human cutaneous melanocytic lesions (22) a possible role for the PA system in melanoma tumor progression. u-PA and PAI expression was related with high metastatic capacity of melanoma cell lines in nude mice (16). This was reflected in the in vivo situation in fresh human melanocytic lesions: u-PA, PAI-1 and PAI-2 but also t-PA and u-PAR emerge in late stages of cutaneous melanocytic tumor progression (22).

In this study, we extend our investigation on the involvement of the PA system in melanoma tumor progression to melanoma of the uvea. Melanoma of the uvea is the most common primary intraocular malignancy in adults (27). Several factors have been found to influence the prognosis. These include tumor cell type (spindle cell type has a better prognosis than non-spindle cell type) and tumor diameter. Uveal melanoma differs from cutaneous melanoma in several aspects. Uveal melanomas spread hematogenously, preferentially to the liver, whereas cutaneous melanomas primarily metastasize lymphogenic. The estimated 5-year-survival rate for uveal melanoma is 75 % (28) and comparable with cutaneous melanoma (29). The aim of this study was to investigate the expression of the different components of the PA system in uveal melanoma. The presence of these proteins was studied on fresh human lesions of primary uveal melanoma and metastases of uveal melanoma using immunohistochemistry and in situ zymography.

Material and Methods

Tissue Specimens

Specimen from primary uveal melanomas were obtained from 45 patients treated at the Department of Ophthalmology, Erasmus University Rotterdam, The Netherlands, between 1987 and 1992. The enucleated eyes were transported on melting ice to the Department of Pathology. After transillumination, the eyes were cross-sectioned through the tumor and part of the tumor was snap frozen in OCT compound (Tissue-tek) and stored at -70 °C. The remainder of the eye was fixed in formalin and embedded in paraffin.

Five uveal melanoma metastases were from three patients from the skin (n=2), the heart (n=2) and from the liver (n=1). From 4 metastases, no primary tumor was available. From one metastasis, the primary tumor was also included in this study. Metastases were from the Departments of Pathology of Nijmegen (n=4) and Rotterdam (n=1).

Antibodies

Rabbit anti-human t-PA and u-PA polyclonal antibodies were from the Gaubius Institute, Leiden, The Netherlands and have been used in earlier studies (16,22). Monoclonal antibodies against human u-PAR (# 3936) and human PAI-1 (# 380) were purchased from American Diagnostica Inc., Greenwich CT, USA. The rabbit and goat polyclonal antibodies against human PAI-2 were a generous gift from E. Schüler, Behring Werke AG, Marburg, Germany. All antibodies against components of the PA system were used in a previous study (22). In that study, using parallel sections for mRNA in situ hybridization, we established with these antibodies that cells producing the mRNA also contained the protein. Detection of the melanocytic differentiation marker gp-100 with monoclonal antibody NKI-beteb (30) was used to establish the melanocytic origin of the tumors.

Immunohistochemistry

For immunohistochemistry, 4 μm cryostat sections were air-dried overnight at room temperature and stored at -80 °C until use. Dilutions of the antibodies and staining procedure were as used before (22). Stainings with monoclonal antibodies were developed with an ABC technique, polyclonal antibodies with peroxidase labeled anti-rabbit or anti-goat secondary antibody. Bound antibodies were visualized by using 3-amino-9-ethylcarbazole as a substrate for peroxidase. After counterstaining the nuclei with Papanicolaou's Harris solution, sections were mounted with Kaisers glycerin (Merck, Darmstadt,

Germany). A lymph node metastasis from a cutaneous melanoma, previously found to express abundant u-PA, u-PAR and PAI-1 protein, was used as a positive control; for t-PA the staining of blood vessels served as internal control and for PAI-2, staining of sections from fresh human placenta served as positive control. An incubation where the first antibody was omitted, served as a negative control.

Score

For each section, the percentage of positive melanocytic cells was estimated. Each section was assigned to one of the following categories: 0%, 1-5%, 5-25% and 25-100% positivity. Notes were taken of other staining components (fibroblast-like cells, extracellular matrix) among the melanocytic areas. Sections were scored independently by two observers (C.M. M., M.R. v. B.). Discrepancies were found in only a few cases. These cases were re-evaluated jointly until agreement was reached.

In Situ Zymography

The technique has been described in great detail by Sappino et al. (31) and De Vries et al. (22). Briefly, 8 μm cryostat sections were covered with an overlay mixture containing dry milk, plasminogen and agar. At spots where plasminogen activation occurs, lysis of the gel takes place, making the gel transluminent. Addition to the gel of polyclonal antibodies against t-PA or u-PA allows discrimination between the two plasminogen activators. In addition, amiloride also inhibits u-PA specifically. With each incubation session, xenograft lesions of the human melanoma cell lines BLM or MV3, known to express abundant u-PA and hardly any t-PA (16), were included as positive control.

Results

Clinico-Pathological Data

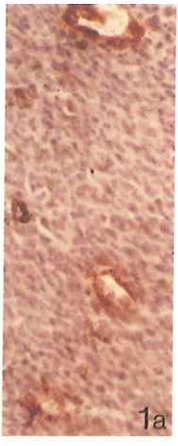
Histopathological diagnosis was obtained on paraffin embedded tissues. Cell type of the tumors was classified with the presence or absence of any epithelioid cells as criterium: spindle (n=14, tumors containing only spindle cells) or non-spindle (n=31, a combination of mixed and purely epithelioid tumors) (32). As other variables we measured: largest tumor diameter; tumor height, presence of episcleral growth, and presence of vascular invasion. All but two specimens examined, contained more than 50% viable tumor cells.
Follow-up was available from all patients included in this study; from one of these patients

a freshly frozen subcutaneous metastatic lesion was obtained. Eight out of 45 patient died due to tumor related death (TRD), 3 died of other causes. The total mean follow-up was 34.7 months.
In 10 eyes extrascleral growth was noted; 3 melanomas showed obvious vascular ingrowth.

Immunohistochemistry
All primary tumors and metastases stained with NKI-beteb (30), recognizing gp-100, a melanocytic differentiation marker (not shown).
Tissue sections were stained for t-PA, u-PA, u-PAR, PAI-1 and PAI-2. Typical examples of immunohistochemical staining are shown in Figure 1. The total number of lesions which stained for the different components are summarized in Table 1. In Figure 2, the percentage of stained melanocytic cells per cell type of uveal melanoma is displayed.

t-PA
In all lesions abundant t-PA immunoreactivity was found in the wall of blood vessels (Figure 1a). The correlation between t-PA expression and cell type is shown in Figure 2: 42 out of 45 tumors contained t-PA, however a moderate to abundant staining (>5 % tumor cells, n=16) was seen predominantly for non-spindle cell type tumors (14/16). Interestingly, in 4 tumors t-PA staining was strikingly in nests of pleiomorphic epithelioid cells. These cells were located perivascular at both invasive fronts, towards the sclera (Figure 1b) and towards Bruch's membrane (Figure 1c). There was no relationship between percentage of t-PA positivity and TRD. All metastases contained t-PA.

Figure 1: Immunohistochemical staining of components of the plasminogen activator system in fresh human uveal melanoma lesions. Typical staining examples in primary lesions (a-c, e-g) and a metastasis (d) are shown. a: t-PA protein in blood vessels. t-PA present in tumor cells at the scleral (Sc) invasive front (b, arrowhead shows a nerve) and in tumor cells adjacent to the retinal pigment epithelium where Bruch's membrane (Br) is ruptured (rupture not seen)(c). d: u-PA is present in tumor cells in this metastatic lesion. e: u-PAR is located at the invasive front towards the sclera in this lesion. f: network like PAI-1 distribution in the extracellular matrix surrounding tumor cells. PAI-2 was found focally in the stroma (g) and in tumor cells (not shown). Cell types: spindle cell tumors (a) and non-spindle cell tumors (b-c, e-g).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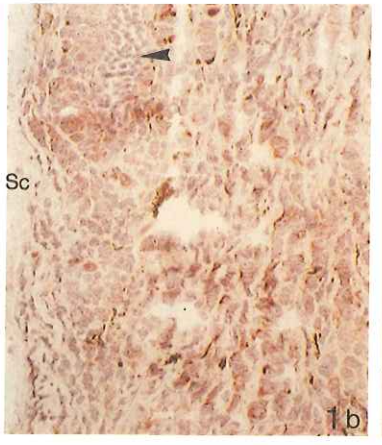
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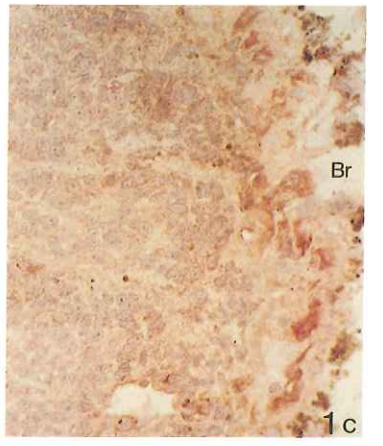
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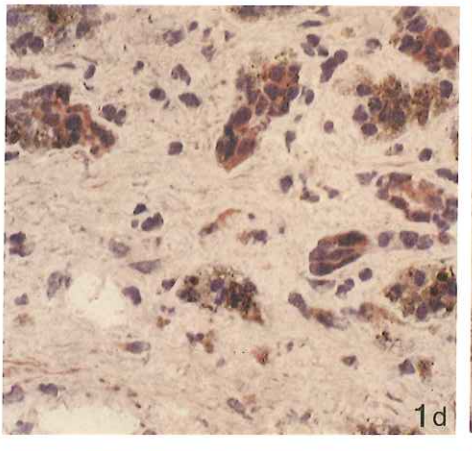
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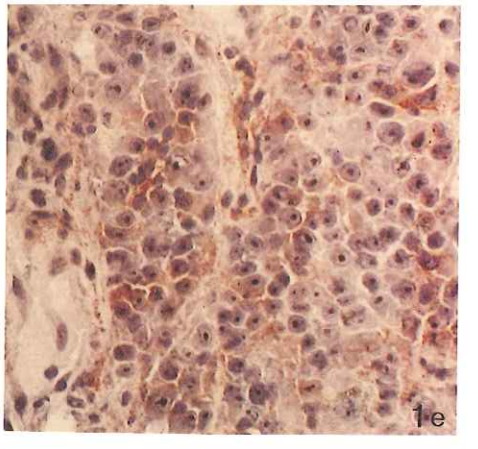
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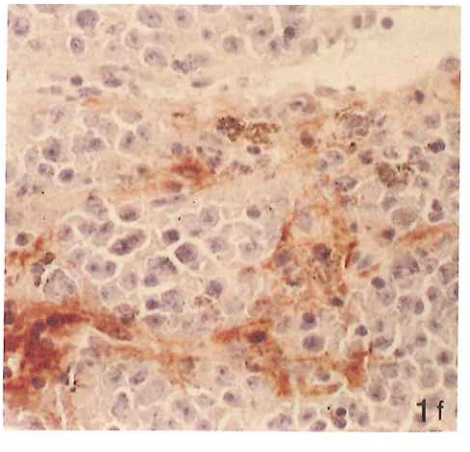
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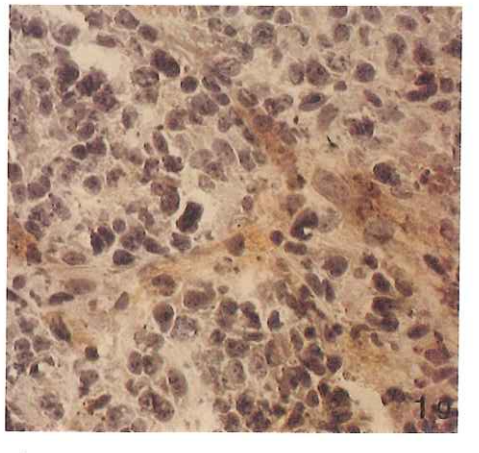
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Table 1 *Immunohistochemical staining of the components of the plasminogen activation system in primary uveal melanomas and in metastases of uveal melanoma.*

	t*	s	e	total
Primary uveal melanoma (n=45)				
Spindle (n=14)				
t-PA	13	3	3	13
u-PA	0	0	0	0
u-PAR	3	1	0	3
PAI-1	2	2	1	3
PAI-2	0	0	0	0
Non-spindle (n=31)				
t-PA	29	16	5	29
u-PA	5	2	0	5
u-PAR	12	5	0	14
PAI-1	1	3	2	3
PAI-2	5	3	1	5
Metastases of uveal melanoma (n=5)				
t-PA	4	1	3	5
u-PA	5	4	1	5
u-PAR	2	0	0	2
PAI-1	3	1	1	4
PAI-2	0	1	0	1

t' = staining of tumor cells; s = stromal cells; e = extracellular matrix stained in these lesions.

u-PA and u-PAR

While no u-PA expression could be detected in any spindle cell type tumors tested, focal u-PA staining (<5%) of tumor cells as well as staining of fibroblast-like cells was detected in 5 non-spindle cell tumors. In 2 cases u-PA expression correlated with TRD, one patient is known to have metastases, and for 2 patients follow-up was short (32 and 18 months). All 5 metastases expressed u-PA protein (Figure 1d).

Focal u-PAR immunoreactivity (<5%) was seen in 15 tumors; 11 out of 15 were non-spindle cell tumors. Both tumor cells (Figure 1e) and stromal cells showed a cytoplasmic staining aspect. Two metastases contained u-PAR.

PAI-1 and PAI-2

PAI-1 protein was observed in only 6 primary tumors (3 were of the spindle cell type), but in the majority of metastasis lesions (4 out of 5). The localization was in tumor cells and in the extracellular matrix (Figure 1f).

PAI-2 protein was observed in a 5 out of 45 tumors and was found only in lesions of the non-spindle cell type. PAI-2 was expressed both by tumor cells and fibroblast-like cells (Figure 1g). One metastasis contained PAI-2.

In 2 out of 5 u-PA positive tumors, u-PAR and PAI were found in the same lesion, both patients died from metastatic disease. One of these two patients showed expression of all five components. A trend towards higher expression (higher percentage of immunoreactive cells) and a higher percentage of positive lesions in non-spindle compared to spindle cell lesions for t-PA, u-PA, u-PAR and PAI-2 can be deduced from figure 2.

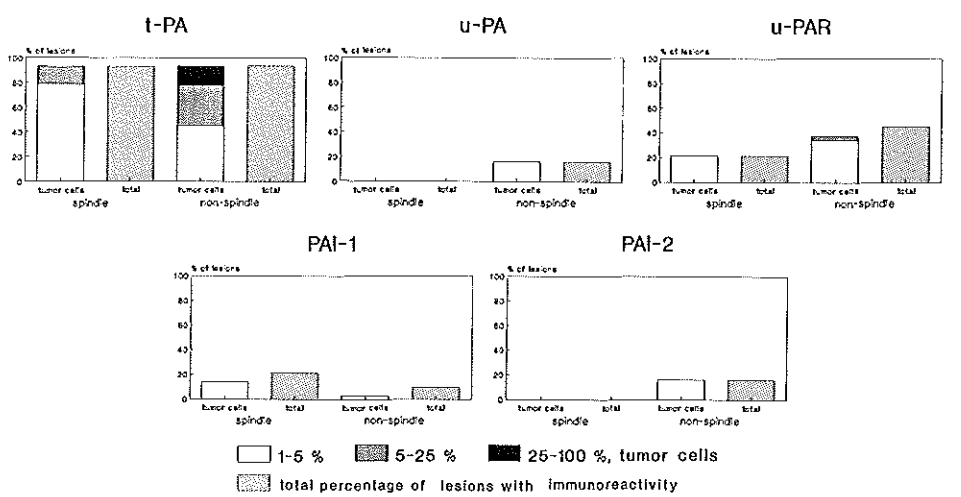

Figure 2. Percentage of immunohistochemically stained tumor cells and percentage immunoreactive lesions including staining of extracellular matrix and stroma cells (e and s in Table 1), expressed as percentage of the total number of lesions. Fourteen tumors had purely spindle cell type (spindle) morphology and 31 tumors (either of mixed origin or purely epithelioid) contained epithelioid cells (non-spindle). In non-spindle cell melanomas, a higher percentage of positive lesions and higher percentages of positive cells is observed for t-PA, u-PA, u-PAR and PAI-2. Metastases are excluded from this diagram due the low numbers included in this study. See also Table 1.

In Situ Zymography

In situ zymography was performed on sections of all lesions studied. In all cases, t-PA activity was found, often in a dotty pattern (Figure 3, first row). By comparing the lysis pattern with the hematoxylin and eosin stained sections, we could assign t-PA activity to blood vessels. Lysis in tumors with t-PA immunoreactivity in the highest category (25-100 %) occurred faster and more general throughout the section (compare Figure 3, first and second row). Also in a few metastases we could detect t-PA activity in an area with mainly tumor cells (Figure 3, third row). Except for the positive control BLM and MV3 xenografts (Figure 3, fourth row), no u-PA activity could be detected in all lesions studied.

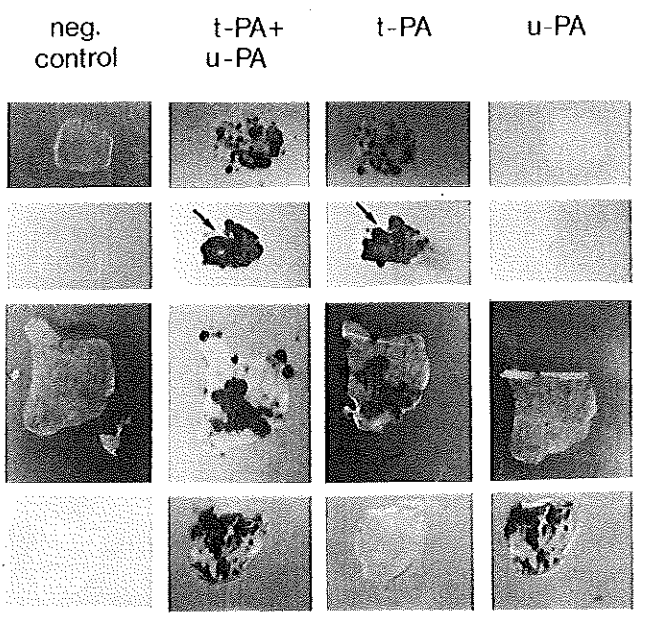

Figure 3: In situ zymography on fresh uveal melanocytic lesions. Plasminogen activation was t-PA mediated in all cases studied and located in blood vessels and in tumor cells. Examples are shown of three cases. One non-spindle uveal melanoma had t-PA immunoreactivity in blood vessels but not in tumor cells (first row). One non-spindle uveal melanoma where 5-25 % of the tumor cells stained for t-PA in immunohistochemistry (second row), note that lysis is more abundant at the sclera site (arrow). Also a metastasis of uveal melanoma (third row) showed more generalized t-PA mediated lysis. A u-PA positive MV3 xenograft, where t-PA mediated lysis had not yet occurred, is included (fourth row). Columns: neg. control: casein layer without plasminogen; u-PA + t-PA: casein layer with addition of plasminogen; t-PA: casein layer + plasminogen + polyclonal antibody against u-PA; u-PA: casein layer + plasminogen + polyclonal antibody against t-PA. Incubation times at 37 °C: 5 hours in all uveal melanoma cases (top three rows) and one hour (fourth row).

Discussion

We recently studied the involvement of plasminogen activation in melanoma tumor progression both in a nude mouse system and in the in vivo situation on sections of fresh human cutaneous melanocytic lesions. u-PA, PAI-1 and PAI-2 expression in human melanoma cell lines correlated with a high metastatic capacity in nude mice. All cell lines contained t-PA and u-PAR (16). In human cutaneous melanocytic lesions, u-PA, PAI-1 and PAI-2 but also t-PA and u-PAR were found in the most malignant lesions only (advanced primary melanomas and melanoma metastases) (22). Here, we extended our study on the involvement of the plasminogen activation system in melanocytic lesions to uveal melanoma. Once the diagnosis of metastatic disease in uveal melanoma is made, (usually by fine needle aspiration), the following median survival is only 2-4 months (33,34). Therefore metastatic tissue is hard to access and only five metastases were studied. Little is known about the presence of proteases in uveal melanoma lesions. The involvement of proteases in metastatic spread of uveal melanoma has recently been suggested by Cottam et al. (35), who detected the 72 and 92 kD type IV collagenase in culture medium of 15 primary cultures of uveal melanomas. Furthermore, t-PA activity in supernatants of primary cultures of uveal melanoma seemed to correlate with scleral invasion in the tumor lesion, whereas no u-PA activity could be detected (36). Interestingly, the choroid itself expresses t-PA (37) and u-PA (38).

In our study, t-PA protein was expressed by tumor cells in the vast majority of the lesions. Expression of t-PA in uveal melanoma therefore does not differ from previous reports on melanoma as a source for t-PA: abundant t-PA was found in melanoma cell lines (16,26,27) and in cutaneous melanocytic lesions (26). t-PA was expressed in a larger percentage of the tumor cells in non-spindle cell tumors. Immunoreactive cells often had a perivascular localization. Also, the localization of t-PA positive cells at both the scleral and Bruch's membrane invasion front was remarkable. This observation is in agreement with Cottam et al. (36), who found that elevated t-PA activity in primary cultures of uveal melanoma correlated with scleral invasion. t-PA positivity was noted in varying percentage (0% up to 25-100% categories) in patients which already died from TRD as well as in patients which were still alive. No relationship between extent of t-PA positivity and TRD could be established.

u-PA expression was found focally in only 5 primary uveal melanomas. Interestingly, all these lesions were of the non-spindle type. In 2 cases u-PA expression correlated with

TRD, one patient is known to have metastases and for two patients, follow-up was relatively short. Also, all metastases contained u-PA positive cells. Therefore, a clear association with progression of disease exists, as could be found for some other types of tumors (17, 19-21), including cutaneous melanoma (22). Furthermore, u-PA positivity might be an additional prognostic marker for uveal melanoma. Nevertheless, six cases with TRD did not display u-PA positivity. Therefore, though the relation between u-PA positivity and progression of disease could be demonstrated in this study, a more extensive study has to be performed to determine whether u-PA is a valuable prognostic marker for uveal melanoma.

With in situ zymography, we were only able to detect t-PA (both blood vessel and tumor cell associated) and no u-PA activity, which is in agreement with findings in primary cultures of uveal melanoma (36). Apparently, the u-PA detected by immunohistochemistry is either of the inactive pro-form or inactivated by PAI. This view is supported by the fact that the staining was only cytoplasmic, where u-PA is normally in the inactive form. Also, the in situ zymography with region sensitivity rather than cellular sensitivity, might be too insensitive to detect less than 5 % scattered u-PA positive cells.

The expression of u-PAR in by far more lesions than u-PA is remarkable, though it is in agreement with our earlier findings in human melanoma cell lines (16), where all cell lines, including the non-metastatic ones, expressed u-PAR. u-PAR was found cytoplasmic, indicating that at least a portion of u-PAR was not available for binding to pro-u-PA (10). Receptor bound pro-u-PA is more efficiently converted to active u-PA (6,12).

For t-PA, u-PA, u-PAR and PAI-2, a higher expression (higher percentage of staining as well as a higher number of lesions involved) was found in the non-spindle cell uveal melanomas (Figure 2) as compared to the spindle cell melanomas which have a better prognosis. These observations and the fact that these components were also frequently detected in metastases, suggests a tendency with progression of disease in this study. Surprisingly, though 4 out of 5 metastases were positive, in primary tumors, this tendency could not be established for PAI-1, which is a prognostic marker for mammary carcinoma (18).

By comparing the involvement of the PA system in uveal and cutaneous melanoma (22), we can summarize the following differences: 1) Using the same polyclonal antibody, t-PA was found in almost all primary and metastatic lesions of uveal melanoma, whereas in cutaneous melanoma, only a few metastases contained t-PA positive tumor cells. 2) In cutaneous melanoma, lesions expressing u-PA expressed also u-PA's regulators u-PAR and

PAI. For uveal melanoma, this co-expression was not as profound (Figure 2). 3) Uveal melanomas express u-PA, PAI-1 and PAI-2 in approximately 10 % of the lesions, whereas in cutaneous melanoma approximately 60 % of the thicker primary lesions were positive. In addition, less tumor cells stained in uveal immunoreactive lesions (all u-PA, PAI-1 and PAI-2 positive lesions were scored in the 1-5 % category). Similar to cutaneous melanoma, u-PA is associated with progression of disease in uveal melanoma. In uveal melanoma, other proteolytic systems could be of more importance in metastatic spread, compared to cutaneous melanoma.

From our study, we conclude that in uveal melanoma, u-PA expression may be associated with metastatic disease and accordingly with poor prognosis. In our opinion, it would be worthwhile to extent research on the presence of u-PA in uveal melanoma to a large group of tumors with known follow-up. In this way, it would be established whether u-PA positivity is of additional prognostic value in uveal melanoma.

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CHAPTER 10

Neural Cell Adhesion Molecule Distribution in Primary and Metastatic Uveal Melanoma

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Summary

Tumor cell adhesion, detachment and aggregation play an important part in tumor invasion and metastasis. Cell adhesion molecules are frequently expressed by tumor cells. It has been demonstrated that cell adhesion molecules of the immunoglobulin superfamily are associated with the development of metastatic behavior in cutaneous melanomas. In order to investigate the role of neural cell adhesion molecule in the development of metastatic behavior, we studied immunohistochemically the expression of NCAM and HNK-1 on a series of primary uveal melanomas and their metastases. We studied 31 primary tumors (among these, 10 were rapidly metastasizing and 16 slowly metastasizing) from 31 patients and 29 metastases from 20 patients. From 13 patients the primary as well as the metastatic tumor were available. HNK-1 was expressed in a lower percentage of all tumors than NCAM. Although NCAM and HNK-1 were expressed in relatively benign, slowly metastasizing primary tumors, we observed an increase of expression in aggressive, rapidly metastasizing tumors, as well as in metastases. There was no similarity between expression of NCAM or HNK-1 in the primary tumors and their corresponding metastases. This implies that cell adhesion molecule expression is not a constitutive characteristic of tumor cells. HNK-1 was negative in 95% of the liver metastases, and negative in 42% of other metastatic sites. The HNK-1 antigen may therefore play a role in the organ specific metastatic behavior of uveal melanomas.

Introduction

Adhesive properties of malignant cells must change repeatedly in order to allow them to detach from their primary location, attach to the extracellular matrix, enter a blood vessel and eventually lodge at a metastatic site.[1] Cell-cell interactions mediated by cell adhesion molecules (CAM) play an important role in these processes.[2] The neural cell adhesion molecule (NCAM) and the intercellular adhesion molecule (ICAM) belong to the immunoglobulin (Ig) superfamily.[2,3] In a variety of human malignancies, tumor progression has been observed to be associated with changes in NCAM expression.[4-6] For cutaneous melanoma it has been demonstrated that the development of metastatic potential is associated with 'de novo' expression of ICAM-1[7-9] and MUC18,[9] an antigen which shows sequence similarity to NCAM.[9,10] In contrast, Denton et al.(1992) found expression of MUC18 on a full range of benign and malignant melanocytic lesions.[11] In uveal

melanomas, melanoma-associated CAM-1 appeared to be negative[12] or poorly expressed[13,14] compared to cutaneous melanomas. ICAM-1 could not be detected in one study,[14] but using a different anti-ICAM-1 monoclonal antibody (MAb) most of the uveal melanomas stained,[13,15] with a preferential reactivity of the mixed and epithelioid cell type.[15] Uveal melanomas metastasize relatively late[16,17] and in contrast to cutaneous melanomas primarily hematogenously, preferentially to the liver. Once hepatic metastases are clinically present, the median survival is extremely poor: only 2-11 months.[18] A role for NCAM in the development of malignant potential of uveal melanomas has so far not been reported.
The purpose of the present study was to investigate if NCAM expression is correlated with the development of metastatic potential in uveal melanoma. We studied primary tumors with known clinical outcome (including rapidly metastasizing and clinically non- or slowly metastasizing tumors) and all available metastases. We report here on NCAM which was stained by a polyclonal antibody, that recognizes all three major NCAM isoforms, and HNK-1, a carbohydrate epitope present on some NCAM species which is stained by the Leu-7 MAb.

Materials and Methods

Patient Selection

From the files of patients with uveal melanoma related death, we were able to collect metastatic tissue from 19 patients (10 liver biopsies, 1 skin biopsy, 1 autopsy, 7 fine needle aspirations). From 13 of these patients the paraffin blocks from the primary ciliary body or choroidal melanoma were available; their follow-up varied between 6 months and 147 months (mean follow-up: 47.4 months). From 5 of these patients paraffin blocks from the primary as well as the metastatic tumor were available. Uveal melanomas create a peak incidence of mortality during the second and third years following enucleation, irrespective of the largest tumor diameter.[19] Tumor related death within 3 years was therefore considered to be due to rapidly metastasizing melanoma (Table 1). Furthermore, we selected 9 patients with a follow-up of at least 10 years after enucleation without clinical evidence of metastatic disease, of whom paraffin blocks of the primary tumor were available. These melanomas were considered to be of low metastatic potential (Table 2). The total mean follow-up of this group was 175.8 months. From nine patients, the paraffin blocks from the primary uveal melanomas and eight corresponding metastases (4 autopsies, 4 biopsies) were obtained from the Eye Pathology Institute in Copenhagen: the follow-up

varied between 2 and 32 years (mean follow-up: 14 years). Three were rapidly metastasizing melanomas(Table 1) and six were of low metastatic potential (Table 2). All these patients died of tumor related death.

Therefore, the total material consisted of 31 primary tumors. Among these, 10 were rapidly metastasizing (Table 1) and 16 slowly metastasizing (Table 2); the remaining five deceased due to uveal melanoma related death between three and 10 years. We investigated 29 metastases (Table 3) from 20 patients. Of 13 patients tissue from the corresponding primary and metastatic tumors were available.

Histology

Sections were cut at 5 to 6 μm and stained with hematoxylin-eosin. Of the primary tumor the predominant cell type (spindle, mixed or epithelioid) and largest tumor diameter ($\leq$10 mm, 10-15 mm, $>$15 mm) were recorded.

Immunohistochemistry

Paraffin sections, 6-7 μm thick were cut and mounted on aminopropyltriethoxysilane (APES, Sigma, St. Louis, USA) coated glass slides and dried overnight at 37°C. After deparaffinizing and rehydrating, endogenous peroxidase activity was blocked by incubation for 20 minutes in methanol containing 3% hydrogen peroxide. The following specific antibodies were used:

1) NCAM: A polyclonal rabbit antibody specific for NCAM was used.[20] This antibody recognizes all three major NCAM isoforms irrespective of the presence or absence of polysialylation.[21] After rinsing the slides in water, antigen retrieval was performed by microwave irradiation (Bio-Rad 37°C, 750 Watt, 2x 5 minutes in 0.1% pronase). The slides were incubated with phosphate-buffered saline (PBS) at 4°C for 10 minutes and subsequently at room temperature (RT) for 5 minutes. The NCAM antibody was incubated for 30 minutes in a dilution of 1:100 at RT. Visualization of antibody binding was performed as described below.

2) The monoclonal antibody HNK-1 (Leu-7), raised against a T cell lymphoma (Becton Dickinson) was used in a dilution of 1:10 and incubated for 30 minutes at RT. The slides were incubated for 30 minutes at RT with biotinylated goat-anti/mouse/rabbit/rat/guinea-pig immunoglobulin (Ig) (Biogenex) in a dilution of 1:50, in PBS with 5% BSA. After washing in PBS/Tween 0.05%, the slides were incubated with the streptavidin-biotin-peroxidase complex (Biogenex) in a dilution of 1:50. The peroxidase was visualized using a

hydrogen peroxide in N-N-dimethylformamide with 3-amino-9 ethylcarbazole dimethylformamide as chromogen substrate. The red stain allowed easy detection of immunoreactivity in pigmented lesions. The sections were counterstained with Mayer's hematoxylin and mounted in glycerin gelatin.

As a negative control, specimens were stained following the same incubation protocol without use of the primary antibodies. A human neuroblastoma served as a positive control for NCAM. For HNK-1, nerve tissue present in the tissue section served as an internal positive control.

NCAM and HNK-1 immunoreactivity were scored semi-quantitatively as percentage of positive cells: score 0: 0%, score 1: 0 - 5%, score 2: 5 - 50%, score 3: 50 - 100%. The immunohistochemical staining was scored without knowledge of the clinical data and was repeated after 2 months. Major (>2 classes) discrepancies did not occur.

Results

Primary Tumors

Thirteen tumors were of the spindle cell type, 14 of the mixed cell type and 4 of the epithelioid cell type. Four tumors were small (≤10 mm), 21 were intermediate size (10-15 mm), and six were large (>15 mm).

NCAM positive tumors showed strong cytoplasmic staining; in three cytoplasmic (Figure 1a) and membrane bound staining was noted (Figure 1b). The results for NCAM expression in the different cell types are illustrated in Figure 2. Of the spindle cell tumors 31% were strongly positive (score 2+3) as were 71% of the mixed cell tumors, and all epithelioid cell tumors. Of the small tumors, 50% were strongly positive for NCAM (score 2+3) as were 53% of the intermediate size, and all large tumors. Of the rapidly metastasizing tumors 70% (Table 1) and of the slowly metastasizing tumors (Table 2), 37.5% were score 2+3.

In the HNK-1 positive tumors, strong cytoplasmic staining was noted (Figure 3).

Of the spindle cell tumors 15% were positive (score 3); of the mixed cell tumors 22% (score 2+3) and of the epithelioid type 25% (score 2). The small tumors were all negative for HNK-1; 14% of the intermediate size and 50% of the large tumors were score 2+3. Of the rapidly metastasizing tumors 30% were positive for HNK-1 (score 2+3, Table 1), whereas 12.5% of the slowly metastasizing tumors were positive (score 3, Table 2).

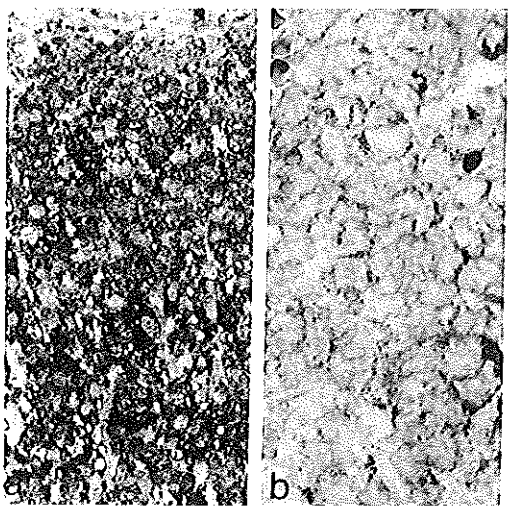

FIGURE 1a and 1b. Field of uveal melanoma of the epithelioid cell type immunostained for NCAM. Cytoplasmic staining is represented in Figure 1a (original magnification x 361), membrane-bound staining in Figure 1b (original magnification x 880). (Hematoxylin counterstain with streptavidin-biotin-peroxidase complex immunoperoxidase with 3-amino-9 ethylcarbazole dimethylformamide substrate).

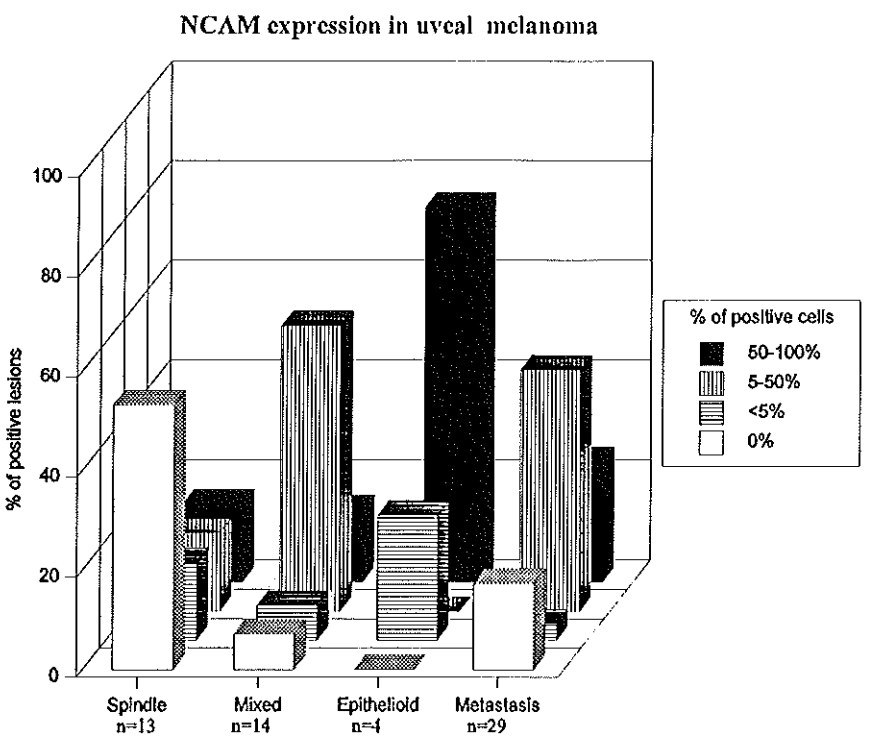

FIGURE 2. Results for NCAM immunostaining in primary and metastatic uveal melanoma.

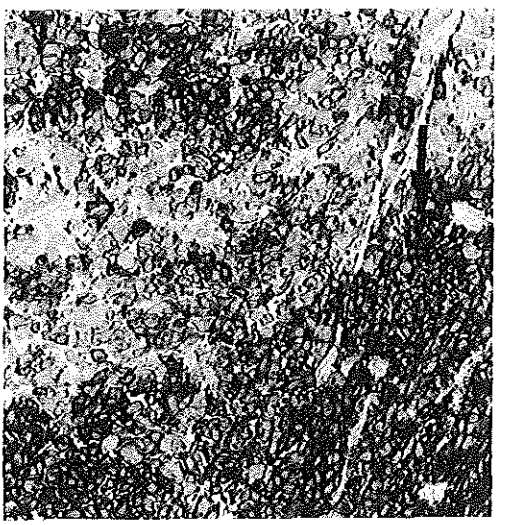

FIGURE 3. Field of metastatic uveal melanoma immunostained for HNK-1. (Hematoxylin counterstain with streptavidin-biotin-peroxidase complex immunoperoxidase with 3-amino-9 ethylcarbazole dimethylformamide substrate; original magnification x 361).

Table 1 *Immunohistochemical data of melanomas metastasizing within 3 years*

Pat. no.	Cell type	Follow-up* (months)	Prim. mel. NCAM	Corr. met. NCAM	Prim. mel. HNK-1	Corr. met. HNK-1
1	S	15	1	n.a.	0	n.a.
2	E	32	2	n.a.	0	n.a.
3	M	27	2	0	0	0
4	M	15	2	n.a.	2	n.a.
5	E	19	2	n.a.	0	n.a.
6	M	15	0	n.a.	0	n.a.
7	E	6	2	n.a.	2	n.a.
8	M	36	0	3	3	0
9	M	30	3	n.a.	0	n.a.
10	M	24	2	n.a.	0	2

**: All patients died of tumor-related death. Prim. mel.: primary uveal melanoma. Corr. met: Corresponding metastasis. E: epithelioid cell type, M: mixed cell type, S: spindle cell type. n.a.: paraffin block not available. Score 0= negative; score 1= 0-5% tumor cells positive; score 2= 5-50% tumor cells positive; score 3= 50-100% tumor cells positive.*

Table 2 *Immunohistochemical data of melanomas with a follow-up of > 10 years*

Pat. no.	Cell type	Follow-up (months)	Prim. mel. NCAM	Corr. met. NCAM	Prim. mel. HNK-1	Corr. met. HNK-1
1	M	224	2	*	0	*
2	S	223	0	*	0	*
3	S	228	0	*	0	*
4	S	209	0	*	0	*
5	S	165	0	*	0	*
6	S	182	0	*	0	*
7	S	181	2	*	0	*
8	M	125	0	*	0	*
9	S	151	1	*	0	*
10	S	147	2	n.a.	3	n.a.
11	M	130	1	3	0	0
12	S	192	3	2	3	2
13	S	300	0	2	0	3
14	S	192	3	2	0	0
15	S	384	0	3	0	0
16	M	216	2	3	0	0

*Prim. mel.: Primary uveal melanoma; corr. met.: corresponding metastasis. *: patients alive and free of metastatic disease after > 10 years; : tumor-related death after > 10 years. S: spindle cell type, M; mixed cell type, E: epithelioid cell type. n.a.: paraffin block not available.*

score 0= negative; score 1= 0-5% tumor cells positive; score 2= 5-50% tumor cells positive; score 3= 50-100% tumor cells positive

Table 3 *Staining pattern in the metastases*

	NCAM					HNK-1				
	neg.	#1	#2	#3	ratio pos.	neg.	#1	#2	#3	ratio pos.
Liver	3	1	6	5	12/15	14	-	1	-	1/15
Lung	1	-	6	2	8/9	5	-	3	1	4/9
Skin*	1	-	2	-	2/4	1	1	2	-	3/4
Abdomen*	-	-	-	-	0/1	-	-	-	1	1/1
Total*	5	1	14	7		20	1	6	2	

*#: score. Score 1 = 0-5% positive tumor cells, score 2 = 5-50% positive tumor cells, score 3 = 50-100% positive tumor cells. *: one metastasis from the skin and one metastasis from the abdomen were unclassifiable for NCAM. Ratio pos.: ratio positive lesions/total lesions metastatic site.*

Metastases

The staining pattern of all metastases for NCAM is illustrated in Figure 2; the staining pattern in the different organs is specified in Table 3. For NCAM, 72% of the metastases stained (score 2+3). For HNK-1, 27% of the metastases stained (score 2+3). Of the 15 liver metastases, only one was positive for HNK-1 (Table 3), whereas 42% of the other metastatic sites were negative. In 5 autopsies, different metastases from the same subject had a varying score (from negative to score 3) both for NCAM and HNK-1.

Corresponding Primary and Metastatic Tumors

Of 13 tumors pairs (partly reflected in Table 1 and 2), 8 primary tumors and their metastases showed positive staining; in 5 major (negative versus positive) discrepancies between the primary tumor and the metastases were noted.

Relationship Between HNK-1 and NCAM

With one exception, all HNK-1 positive primary and metastatic tumors were also positive for NCAM. However, 71% of the primary tumors were NCAM positive, whereas only 20% were HNK-1 positive. Similarly, of the metastases 72% were NCAM positive and 27% were HNK-1 positive.

Discussion

The percentage of NCAM positive tumors in our study was higher than has been reported for cutaneous melanomas.[5,11,22] This might be explained in terms of methodological differences: we used a different (polyclonal) antibody on formalin-fixed, paraffin embedded tissue and also applied an antigen retrieval method. NCAM immunostaining was mostly cytoplasmic and less frequently both cytoplasmic and membrane-bound, which is in keeping with previous investigations.[5,22]

HNK-1 was expressed in a lower percentage of the tumors than NCAM, but was rather consistently expressed in NCAM positive tumors, primary as well as metastatic. NCAM expression was noted in a high percentage of liver metastases as well as in lung and pleural metastases. Interestingly, 95% of the liver metastases were negative for HNK-1, in contrast to other metastatic sites. These findings might suggest a possible role for the HNK-1 antigen in the organ-specific pattern of uveal melanoma metastasis.

We found an increase in NCAM and HNK-1 staining in lesions with epithelioid cells (mixed and epithelioid cell type). This is in concordance with the findings for cellular adhesion molecule ICAM-1 in primary uveal melanoma.[13,15] The presence of epithelioid cells is one of the factors associated with progression of uveal melanomas (development of metastatic potential).[23] Furthermore, we found an increase in NCAM and HNK-1 staining in large tumors, in rapidly metastasizing tumors, and in metastases. NCAM and HNK-1 expression in the primary tumors did not correspond with that in the paired metastases, indicating that NCAM and HNK-1 expression are not a constitutive characteristic of the tumor cells. Immunohistochemical studies on the relationship of NCAM expression with tumor cell typing are rare.[5-6,11,21,22,24-26] The antibody to NCAM we used in our study has been investigated on cell lines of human small cell lung cancer,[27] glioma cells[28] and murine melanoma cells.[29] In these cell lines it has been found that metastasizing cells express less NCAM than non-metastasizing cells,[28,29] which contrasts with our findings on formalin-fixed, paraffin embedded tissue. However, we found that NCAM and HNK-1 expression in 3 uveal melanoma cell lines did not correspond with that in the tissue from which the cell lines were derived (data not shown). This indicates that expression of cell adhesion molecules may be modulated by the tumor cell microenvironment. Alternatively, tissue processing might interfere with NCAM immunoreactivity.

That NCAM is expressed in epithelioid cells seems surprising because epithelioid cells are morphologically non-cohesive. It is not clear how adhesion molecules on the surface of

cancer cells influence metastasis. Adhesion molecules may delay the escape of tumor cells from the primary site due to an increased adhesion to other cells and to intercellular matrix proteins. However, an altered pattern of CAM expression or expression of aberrant CAM might disrupt normal adhesion and attachment of these cells to a blood vessel or a metastatic site.[4]

NCAM exhibits special carbohydrate characteristics: glycosylation of NCAM seems to be regulated during development and to influence the adhesive function of the molecule.[30] It has been suggested that polysialylated NCAM present in early (embryonic and fetal) stages of development is involved in cellular migration, whereas the expression of unsialylated NCAM in tissues may be important for local differentiation and organization.[31] Polysialylation of the NCAM molecule decreases its adhesion properties and may therefore play a role in connection with tumor invasion and metastasis. Immunohistochemical investigation gives information about the subcellular localization of NCAM, but does not enable distinction between the different isoforms, neither does it provide information about NCAM polysialylation.

In summary our results show that expression of NCAM and to a lesser extent of HNK-1 is associated with the development of malignant potential of uveal melanoma. The prognostic value of NCAM and HNK-1 expression in uveal melanoma remains to be established.

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CHAPTER 11

Considerations

Summary

Samenvatting

List of Publications

Dankwoord

Curriculum Vitae

Considerations

Approximately 40% of the patients with ciliary body and choroidal melanoma who undergo enucleation die within 10 years after diagnosis of metastasis.[1] Nevertheless, clinically evident metastatic disease at the time of initial presentation of the primary tumor is uncommon, metastases being detected in only 1 to 2% of patients; furthermore, death due to local recurrence is extremely rare. These findings imply that subclinical metastatic disease is more common than previously assumed. There is currently no effective therapy once clinically detectable metastases have developed. Metastatic dissemination in uveal melanoma is almost exclusively hematogenous, preferentially to the liver. The development of metastatic disease subsequent to surgical removal of the primary tumor implies that undetectable micrometastases must be present at initial presentation already. Identification of patient subgroups with increased risk for the development of metastases might be important when effective adjuvant treatment for micrometastases would become available. Against this background we made an effort to identify prognostic factors which would help to recognize patients at high risk of developing metastatic disease.

Prognostic Parameters

Histologic cell type and largest tumor diameter (LTD) have traditionally been regarded as the leading predictors of survival. Ideally, histopathological prognostic parameters should be simple to assess, reproducible, and amenable to analysis in conventionally processed tissue specimens. Morphological cell typing of uveal melanomas is however, subjective to variations in interpretation. Cell typing according to the modified Callender classification[2] appeared no longer as an independent prognostic parameter after multivariate analysis in several studies.[3-6] Therefore, Callender's classification has been further modified into a two-category system: tumors with and tumors without epithelioid cells.[3] The prognostic value of this system needs to be investigated further. More objective classification parameters have emerged from cytomorphological and DNA flow-cytometrical studies. We have demonstrated that DNA-ploidy and cell type are strongly correlated. Furthermore, we found that DNA-ploidy and LTD appeared to be the most significant parameters in predicting clinical outcome. In most studies LTD appears as one of the most significant and reproducible parameters in predicting survival. Although, the sensitivity of LTD as prognostic parameter has been reported to be 76%, the specificity was only 44% which

allows for a 56% chance of a false prediction of outcome.[6]

It has been calculated that the median survival time for patients with an LTD of 10 mm is substantially longer than the median survival time of patients with LTD of 20 mm and the linear doubling time from the primary tumor, estimated on clinical data, suggest.[1,7] This implies that as tumors grow, they progressively disseminate progressively more rapidly proliferating cells, which further attenuates the inverse relationship between LTD to survival time. Tumor height or extrascleral growth have not been reported as independent prognostic parameters in recent studies on multivariate analysis of prognostic factors. The TNM classification of uveal melanomas might provide a model of progression in combining LTD, tumor height and scleral invasion in the T category. However, in our study of DNA-ploidy, T classification did not appear to be of prognostic significance.

In a clinical study, it has recently been demonstrated that delayed treatment of selected small and dormant appearing choroidal melanomas does not substantially increase the probability of melanoma-specific mortality.[8] Nevertheless, even small or pure spindle cell melanomas may give rise to metastases: although for tumors < 7 mm death to metastatic disease is only sporadically reported,[9] the 5-year mortality rates in patients with melanomas < 10 mm is reported to be 16%.[10] Therefore, prolonged clinical observation of melanomas from 7 mm to 10 mm diameter is doubtful.

Fine needle aspiration of intra-ocular tumors has proven to be a safe and useful technique[11] and appears to be reliable in distinguishing between melanomas and metastatic lesions and other primary tumors. However, the cytological differentiation between nevi and small melanomas remains a problem. Techniques such as DNA-ploidy measurements and determination of proliferative activity can be applied to fine needle aspirates in order to select from patients with clinically small tumors and therefore with an overall low risk for metastatic disease, those with a high risk and a need for further treatment. Furthermore, these techniques can be applied to fine needle aspirates from iris and/or ciliary body tumors, which clinically give rise to problems in differentiating between nevi and melanomas.

The presence of closed vascular loops has been proclaimed the parameter most significantly associated with tumor related death of all variables tested.[3,12,13] However, the reproducibility of the differentiation between the various vascular patterns and the prognostic value of this parameter remains to be proven by independent additional studies.

It is apparent that important trends have emerged from retrospective prognostic studies. In a prospective study on a large series of uveal melanomas the prognostic value of

histopathological covariates like cell type, LTD, the vasculature pattern, the Mib-1 index, c-myc oncoprotein and possibly DNA-ploidy and nucleolar measurements like standard deviation of the nucleolar area[14] needs to be further investigated. The Mib-1 index and c-myc oncoprotein can currently be routinely determined by immunohistochemistry. Thus, a combination of prognostic parameters might be selected with high sensitivity and specificity. If effective systemic treatment for metastatic uveal melanoma would be available, such an approach might provide a way of selecting patients at high risk of developing metastatic disease for adjuvant therapy.

Tumor Progression

At present, we know very little about the events which initiate the development of uveal melanoma or about the factors which promote their metastatic dissemination. The acquisition of this knowledge will provide us with more accurate indices of survival and a better prospect for cure.

Based on clinical and histopathological studies, it has been proposed that tumor progression in cutaneous melanoma is a process of distinct steps, leading from a 'promoted melanocyte' to a metastatic melanoma (Table 1),[15] although for each tumor not every step necessarily needs to be taken.

Table 1 *Proposed Tumor Progression in Cutaneous Melanoma.**

Step	Melanocytic lesion
1)	Common acquired and congenital nevus
2)	Dysplastic nevus
3)	Primary melanoma, radial growth phase
4)	Primary melanoma, vertical growth phase
5)	Metastatic melanoma.

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For uveal melanoma, a similar model might be considered. Thus, for the different steps the role of various factors that play a role in the acquisition of metastatic potential might be investigated, which could open new ways to interfere with this process.

In uveal melanoma progression, the steps can be proposed by histological cell type (Table 2) or by LTD (Table 3). However, precursor lesions of uveal melanoma (choroidal nevi or atypical melanocytic hyperplasia) are rarely available for histopathological studies. In this

respect it seems important to obtain choroidal nevi, either from autopsies or from incidentally detected nevi in enucleated blind and painful eyes. Furthermore, a pre-invasive, intra-epithelial (retinal pigment epithelium) precursor lesion has not been demonstrated in uveal melanomas. The advantage of LTD as a model of progression is that the different steps are highly reproducible and highly significant in predicting clinical outcome.

Table 2 *Proposed Tumor Progression in Uveal Melanoma.*

Step	Melanocytic lesion by cell type
1)	Choroidal nevus
2)	Primary melanoma, spindle cell type
3)	Primary melanoma, mixed cell type
4)	Primary melanoma, epithelioid cell type
5)	Metastasis

Table 3 *Proposed Tumor Progression in Uveal Melanoma.*

Step	Largest tumor diameter
1)	≤ 7 mm
2)	7 - 10 mm
3)	> 10 mm - 15 mm
4)	> 15 mm
5)	Metastasis

A vertical growth phase is not recognized in uveal melanoma. However, at a certain point uveal melanomas invade the sclera or break through Bruch's membrane, grow into the retina and through the internal limiting membrane into the vitreous. We have investigated the role of cell-matrix interaction and found that the plasminogen activator system and neural cell adhesion molecule distribution play a role in the progression of uveal melanomas as defined by cell type. The prognostic value of these parameters and a possible role of other superfamilies of adhesion molecules in tumor progression remains to be established. Furthermore, we found that the Mib-1 defined proliferative index and the regulating oncoprotein c-myc are independent prognostic parameters. The Mib-1 index correlated with the presence of epithelioid cells, but a relationship between these parameters and LTD could not be established. Oncogene activation (i.e. N-*ras* mutations)

could not be demonstrated primary uveal melanomas.

It seems likely that vascular invasion is a major step in the progression of uveal melanoma. Tumor classification of uveal melanocytic lesions, based on the microcirculation architecture by light and electron microscopy has been proposed: nevi had a characteristic vascular architecture and melanomas that have the same vascular profile had a very high probability to cause an intermediate biological behavior. Melanomas with closed vascular networks had a very high probability to cause death due to metastatic disease.[16] However, nevi are rarely available for histological examination. Therefore, the vascular pattern of uveal nevi is of little practical value as point of reference.

A further goal of investigation may be the reproducibility of the prognostic value of the vascular pattern and the development of the tumor vasculature, in particular closed vascular patterns, by studying the interaction between uveal melanoma cells and the extracellular matrix. In particular the role of growth factors and their receptors in the angiogenesis of uveal melanoma needs to be elucidated.

In Vitro Models

In this models of uveal melanoma a major advance in research has been the development of culture techniques that allow the establishment of cell lines. When cell lines can be established from uveal melanomas, defining the steps between benign melanocytes and metastatic uveal melanoma, several biological and molecular properties might be studied in detail. Although such model systems do not necessarily reflect the situation in vivo, they can be helpful in determining the role of carcinogenesis related events, like abnormalities in cancer genes, autocrine and paracrine growth-stimulation, inhibition of apoptosis, cell adhesion and cell migration related molecules and matrix proteases in melanoma progression. Furthermore, elucidation of the molecular basis of the recognition of malignant cells by the host immune system could lead to the establishment of useful targets for specific immunotherapy of uveal melanoma.

Animal Models of Human Uveal Melanoma

In order to develop new techniques for the systemic treatment of uveal melanoma, knowledge about the growth and organ specific metastasis of these tumor cells is a prerequisite. Transplantation of human uveal melanoma cell lines into animal eyes has been used to study tumor growth and metastasis. It was established that in nude mice the success rate of xenografting of primary uveal melanoma tissue is low, and spontaneous metastases

from xenografts are rare.[17,18] In immunosuppressed rabbits successful heterotransplantation has been described.[19,20] Recently, we developed a chicken embryo model to study the growth of human uveal melanoma.[21] Further investigation of reproducible animal models is warranted.

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Summary

The aim of this thesis was the identification of reliable progression parameters as prognostic markers in primary uveal melanoma, focussing on clinicopathological characteristics.

General background information on epidemiology, histologic classification, tumor grading, the survival and therapy of uveal melanoma patients is given in Chapter 2. The difficulties in reproducing the subjective histologic classification encouraged research on more objective parameters. From our review on advances in research on prognostic parameters (Chapter 3) new parameters appeared in the field of cytomorphometry (standard deviation of the nucleolar area and mean of the ten largest nucleoli) and DNA analysis (ploidy studies). From recent clinicopathologic studies it appeared that the tumor vasculature (presence of closed vascular patterns) is one of the most significant factors in predicting metastatic potential of uveal melanomas. Reviewing the relevant literature on identification of characteristics in the uveal melanoma genotype and phenotype revealed considerable differences in the field of cytogenetics in cutaneous and uveal melanomas. Furthermore, the specific surface phenotype of melanoma-associated antigens revealed marked differences between these tumors. So far, limited research has been performed on cell-cell and cell-matrix interaction, again indicating differences in uveal and cutaneous melanomas. This led to the conclusion that some of these findings may relate to the difference in biological behavior between these tumors.

Progression in tumors can be defined as irreversible acquisition of metastatic potential. The benign or precursor lesions of uveal melanoma progression are difficult to obtain. In Chapter 4 we describe a rare case of -paraneoplastic- bilateral melanocytic hyperplasia which led to bilateral uveal melanomas. Our research in the field of the genotype of uveal melanoma revealed no mutations in the N-*ras* gene (Chapter 5). These mutations have been demonstrated in cutaneous melanomas and were attributed to the effect of UV radiation. Ki-67 defined proliferative activity was performed by immunohistochemistry on a series of uveal melanomas (Chapter 6). We compared melanomas, which had received irradiation prior to enucleation with non-irradiated melanomas. Our findings revealed that uveal melanomas which had not been irradiated prior to enucleation, had a significant higher proliferation rate. An association with clinico-pathologic parameters could not be demonstrated. To address the striking variation of aneuploidy reported for uveal melanomas

we performed DNA flow cytometric analysis on a series of melanomas (Chapter 7). A strong correlation between DNA-ploidy and cell type, but not with other clinicopathological parameters was demonstrated. Furthermore, we found that pre-enucleation irradiated melanomas were significantly more aneuploid than non-irradiated melanomas. DNA-ploidy and LTD were the most significant factors, in predicting clinical outcome. We studied retrospectively indices of proliferation, such as mitotic count and the Mib-1 (Ki-67) index on a series of uveal melanomas and compared their prognostic significance with clinicopathologic parameters. Along the same line we investigated the expression of the regulating proteins c-myc and Bcl-2 (Chapter 8). We found that LTD, cytoplasmic expression of the c-myc oncoprotein and the Mib-1 index were useful as independent prognostic parameters. The strong inverse relationship between the oncoproteins c-myc and Bcl-2 indicate that c-myc and Bcl-2 opposite in immortalizing uveal melanoma cells. Degradation of the extracellular matrix and other tissue barriers is a prerequisite in the acquirement of malignant potential. An important proteolytic system involved in tumor progression comprises the proteins of the plasminogen activator (PA) system. We investigated the expression and distribution of the various components of the PA system and the presence of PA enzyme activity (Chapter 9). Although we found that urokinase-PA expression correlated with occurrence of metastasis, the prognostic value of u-PA remains to be established. At the invasive front (towards the sclera and Bruch's membrane) tissue-type PA was markedly present. The different components of the PA system were markedly less expressed compared to cutaneous melanomas. In Chapter 10 we investigated the role of neural cell adhesion molecules (NCAM) in the development of metastatic behavior in a series of primary and metastatic uveal melanomas. We found that NCAM and to a lesser extent HNK-1 is associated with the development of malignant potential. The prognostic significance of these parameters remains to be established. There was no similarity between expression of NCAM or HNK-1 in the primary tumors and their corresponding metastases, implying that cell adhesion molecule distribution is not a constitutive characteristic of tumor cells. HNK-1 was negative in 95% of the liver metastases. The HNK-1 antigen may play a role in the organ specific metastatic behavior of uveal melanomas. In Chapter 11 we propose a model for progression in uveal melanoma, based on cell type or tumor diameter.

Samenvatting

Het doel van dit onderzoek was het identificeren van betrouwbare progressie parameters als prognostisch merkteken in het primaire melanoom van de uvea, vooral gericht op klinisch-pathologische parameters. Algemene achtergrondinformatie over de epidemiologie, de histologische classificatie, de tumor gradering, de overleving en de therapie wordt gegeven in Hoofdstuk 2. De moeilijkheden bij het reproduceren van de subjectieve histologische classificatie hebben geleid tot onderzoek naar meer objectieve parameters. Uit onze naspeuringen naar de voortgang in het onderzoek over nieuwe prognostische parameters blijkt dat op het gebied van de cytomorfometrie (de diameter van de tien grootste nucleoli en de standaard deviatie van het nucleolus oppervlak) en DNA analyse (ploidie studies) nieuwe parameters werden gevonden. Recente klinisch-pathologische onderzoeken wezen op het belang van de tumor vasculatuur (de aanwezigheid van gesloten vaatpatronen) als een van de prognostische factoren, welke het meest significant bleek in het voorspellen van het metastaserend gedrag van het uveamelanoom. Bij het vergelijken van de relevante literatuur op het gebied van de cytogenetica blijkt dat er op dit gebied aanzienlijke verschillen zijn met het huidmelanoom. Verder zijn er verschillen aangetoond in het phenotype van melanoom-geassocieerde antigenen. Tot heden is er weinig onderzoek verricht op het gebied van cel-cel en cel-matrix interacties. Hierbij werden echter ook ook verschillen gevonden tussen het huidmelanoom en het uveamelanoom. Hieruit kan geconcludeerd worden dat deze verschillen waarschijnlijk gerelateerd zijn aan het verschil in biologisch gedrag tussen deze tumoren.

Progressie in tumoren kan gedefinieerd worden als voortdurende toename in graad van kwaadaardigheid. Het is moeilijk om benigne of voorloperlesies van het uveamelanoom te verkrijgen. In Hoofdstuk 4 beschrijven wij een zeldzaam geval van -paraneoplastische- bilaterale, melanocytaire hyperplasie waaruit een bilateraal uvea melanoom is ontstaan. Uit ons onderzoek op het gebied van het genotype van het uveamelanoom is gebleken dat er geen puntmutaties in het N-*ras* gen konden worden aangetoond (Hoofdstuk 5). Deze mutaties zijn echter wel gevonden in het huidmelanoom en werden toegeschreven aan het effect van ultraviolet straling. In een serie huidmelanomen werd de -als Ki-67 gedefinieerde- proliferatie activiteit bepaald door middel van immunohistochemie (hoofdstuk 6). We vergeleken melanomen, welke voorafgaande aan de enucleatie werden bestraald, met niet-voorbestraalde tumoren. Uit onze bevindingen blijkt dat niet-voorbestraalde tumoren een significant hogere proliferatie activiteit hadden. Een associatie met klinisch-pathologische parameters kon niet worden aangetoond. Om het opvallende

verschil in aneuploidie, beschreven voor het uveamelanoom te onderzoeken, hebben we DNA flow cytometrie verricht (Hoofdstuk 7). Wij vonden een sterke correlatie tussen DNA-ploidie en het celtype, maar niet met andere klinisch-pathologische parameters. Verder vonden wij in voorbestraalde melanomen significant meer aneuploidie dan in niet-voorbestraalde melanomen. De DNA-ploidie en de tumordiameter waren factoren welke het meest significant waren als parameter voor de overleving. We hebben retrospectief proliferatie-markers zoals de mitosenfrequentie en de Mib-1 (Ki-67) index in een serie uveamelanomen onderzocht en de prognostische waarde hiervan vergeleken met klinisch-pathologische parameters. In dezelfde studie onderzochten wij de expressie van de regulerende eiwitten c-myc en Bcl-2 (Hoofdstuk 8). Het bleek dat cytoplasmatische expressie van het c-myc oncoproteine en de Mib-1 index bruikbaar zijn als onafhankelijke prognostische parameters. De sterk omgekeerde correlatie tussen c-myc en Bcl-2 expressie duidt op een samenwerking tussen c-myc en Bcl-2 waardoor melanoomcellen in leven blijven. Degradatie van de extracellulaire matrix en andere weefselbarrieres is noodzakelijk voor het verwerven van metastaseringscapaciteit. Een belangrijk proteolytisch systeem, dat betrokken is bij tumor progressie, omvat de eiwitten van het plasminogeen activator (PA) systeem. We onderzochten de expressie en de verschillende componenten van het PA systeem en de aanwezigheid van PA enzymactiviteit (Hoofdstuk 9). We hebben aangetoond dat urokinase-PA expressie correleert met het optreden van metastasen, hoewel de prognostische waarde van urokinase-PA nog moet worden bepaald. Tissue-type PA was opvallend aanwezig aan het front van tumorinvasie (richting sclera en de membraan van Bruch). De verschillende componenten van het PA systeem kwamen aanzienlijk minder tot expressie dan bij het huidmelanoom. In Hoofdstuk 10 hebben wij de rol onderzocht van neurale cel adhesie moleculen (NCAM) in het ontwikkelen van metastaseringscapaciteit in een serie primaire en gemetastaseerde uveamelanomen. Wij vonden dat NCAM en in mindere mate HNK-1 geassocieerd zijn met het ontwikkelen van metastaseringscapaciteit. Er werd geen overeenkomst gevonden tussen de expressie van NCAM en HNK-1 in de primaire tumoren en hun corresponderende metastasen. Dit houdt in dat expressie van deze celadhesiemoleculen geen essentieel karakteristiek is van de tumorcellen, maar waarschijnlijk beïnvloed wordt door de micro-omgeving. Het HNK-1 antigeen was negatief in 95% van de lever metastasen. Het HNK-1 antigeen kan daarom een rol spelen in het orgaanspecifieke metastaseringsgedrag van uveamelanomen. In Hoofdstuk 11 stellen wij een progressie model op voor het uveamelanoom, gebaseerd op het celtype en de tumor diameter.

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Curriculum Vitae

De schrijfster van dit proefschrift werd geboren op 27 september 1951 te Heerhugowaard. Na haar eindexamen H.B.S.-B aan het Petrus Canisius College te Alkmaar in 1969 volgde zij de School voor Toeristische Vorming in Breda en was enige tijd werkzaam in het toerisme. Van 1972 tot 1979 studeerde zij Geneeskunde aan de Rijks Universiteit Utrecht. Van 1979 tot 1981 was zij als assistent geneeskundige niet in opleiding (AGNIO) interne geneeskunde en cardiologie werkzaam in het St. Elisabeth Gasthuis te Arnhem. Van 1981 tot 1985 werd zij opgeleid tot patholoog aan de afdeling Pathologie van het Academisch Ziekenhuis Nijmegen (opleiders Prof. Dr. P.H.M. Schillings en Prof. Dr. G.P. Vooijs). Deze opleiding werd in 1984 en 1985 voltooid aan de afdeling Pathologie van de S.S.D.Z. (opleider Dr. B. Makkink) te Delft en de laatste maanden aan de afdeling Pathologie, Academisch Ziekenhuis Rotterdam (opleider Prof. Dr. R.O. van der Heul). In 1983 volgde zij een stage pathologie in het Royal Perth Hospital, Perth, W.Australië. November 1985 werd zij als patholoog geregistreerd. Sindsdien is zij parttime (60%) werkzaam in het Academisch Ziekenhuis Rotterdam in dienst van de afdeling pathologie (Prof. Dr. R.O. van der Heul, later Prof. Dr. F.T. Bosman) en de afdeling oogheelkunde (Prof. Dr. P.T.V.M. de Jong). Tot december 1986 bekwaamde zij zich in het deelspecialisme ophthalmopathologie onder leiding van Prof. Dr. W.A. Manschot (Academisch Ziekenhuis Rotterdam) en Prof. Dr. W.R. Lee (Department of Pathology, University of Glasgow, Schotland). Sinds 1987 is zij lid van de Oog en Orbita Tumoren Commissie en in 1990 werd zij gekozen tot lid van de European Ophthalmic Pathology Society. Zij is getrouwd met G.G.C. Groothuizen, oogarts en moeder van Wouter (geboren 1986) en Rolf (geboren 1988).