PRIMARY AND METASTATIC UVEAL MELANOMA:

towards a therapeutic approach

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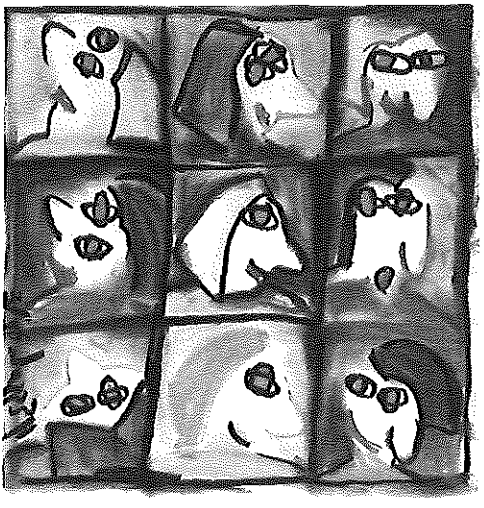

To my dearest Yolanda

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 Clinical aspects of uveal melanoma

1.1.1 Introduction to the subject

Uveal melanoma is a uncommon disease with on estimation 80-100, yearly incident cases in The Netherlands (6 per million) (De Jong, 1987). Nevertheless, uveal melanoma is the most common primary malignant intraocular tumors in adults (Egan, 1988). Moreover, this type of cancer often proves fatal to the patient. Although 50 % of the patients treated for their primary uveal tumor recovers completely, the other 50 % eventually dies of metastatic disease (Gamel,1993; McLean, 1993; Diener-West, 1992; Jensen, 1982). In other words, half of the patients suffering from a primary uveal melanoma develops metastatic disease at a distant site, which is then lethal in all cases. In contrast to cutaneous melanoma, uveal melanoma disseminates primarily haematogenously and with a strong preference for the liver; once metastases have been diagnosed, the patient's life expectancy is only 2 to 9 months (Kath, 1993; Albert, 1992; Gragoudas, 1991; Rajpal, 1983; Bedikian, 1981; Char, 1978). These metastases often become clinically manifest years after initial treatment, that is, uveal melanomas metastasize relatively late. The patient's median survival time after discovery of the primary tumor is 6.5 years (McLean, 1993), with a peak of elanoma-related deaths between the second and fourth year following initial treatment (Zimmerman, 1978). In brief, the studies presented in this thesis basically aim at prevention of (death by) metastatic uveal melanoma. Before we continue, it should be noted that wherever the term uveal melanoma is used in this book, it refers only to melanoma of the choroid and the ciliary body. Melanomas located in the iris, the third part of the uvea, are relatively benign with a low incidence of developing metastases at distant sites and are therefore left out of the study here. The reader interested in iris melanoma is referred to other reports (Grossniklaus, 1995; Jensen, 1993; Brown, 1990).

To date, studies on uveal melanoma mainly focused on the refinement of clinical diagnosis and treatment of the primary tumor. This has resulted in a decrease in the clinical misdiagnosis rate from 3.5 up to 20 % (COMS, 1990; Jensen, 1963) to less than 1 % at present (Davidorf, 1983; COMS, 1990; personal data). The fact that today, in specialized centers, the correct diagnosis of primary uveal melanoma can be made in more than 99 % of the cases is mainly owed to the application of indirect ophthalmoscopy in experienced hands together with the use of ultrasonography. Fluorescence angiography has made the diagnosis also more reliable. These devices are helpful in discriminating uveal melanoma from other types of ocular tumors and disorders, such as choroidal nevi, haemangiomas and metastases, granulomas and macular degeneration. For instance, a tumor of biconvex or mushroom-like shape that shows choroidal excavation on the B-mode and a low to moderate high internal reflectivity on the A-mode in an examination by ultrasonography will, when combined with indirect ophthalmoscopy, certainly be recognized as a choroidal melanoma. However, metastatic choroidal lesions, that usually show a high internal reflectivity on the A-mode, can be highly variable in their ultrasound pattern. In such cases examination of fine-needle aspiration biopsies (FNAB) can be useful (Shields, 1993), but then again, in the cytological evaluation of FNAB, differentiating melanomas and other intraocular tumors can be hard too. Next, in cases in which it is difficult to discriminate a small dormant melanoma from a choroidal nevus, tumor growth can be followed by B-mode ultrasonography to evaluate the tumor height, and by serial fluorescence angiography to evaluate the tumor diameter. Finally, in diagnosing primary uveal melanomas, magnetic resonance imaging with contrast (gadolinium) may be used. The problem with applying this technique, however, is that the characteristical image of the paramagnetic melanin is not present in all cases (Ferris, 1995; De Potter, 1994; Bloom, 1992).

When a primary uveal melanoma has been diagnosed, the patient is usually screened for the presence of extraocular malignancies and distant metastases of the uveal melanoma by a complete physical examination, an X-ray of the chest, liver function tests, and liver ultrasonography. In about 8% of the cases another second primary tumor can be found (Kindy-Degnan, 1989; personal data). Screening of the patient after diagnosis of a primary uveal tumor brings related distant metastases to

light in approximately 2 % of the cases (Pach, 1986; personal data). Furthermore, before the decision of treatment can be made, all factors that may be of importance to the therapeutic choice have to be evaluated for each patient individually, such as visual acuity, intraocular pressure and other concomitant disorders of the affected and the non-affected eye; size, location and episcleral extension of the tumor, and age and general health of the patient (Shields, 1993; id. 1991).

1.1.2 Treatment of the primary tumor

Once the diagnosis of primary uveal melanoma has been made, the choice of treatment is controversial (Shields, 1993; id. 1991). In the past, the only available treatment was enucleation of the tumor-containing eye. It is generally agreed upon that this therapy is still preferable in cases of large melanomas (largest tumor diameter (LTD) > 15 mm and/or tumor height > 5 mm) (De Jong, 1987; Manschot 1980). However, mainly with regard to small (LTD <10 mm) or medium sized (LTD 10 - 15 mm) tumors, newly developed so-called "conservative" treatments as opposed to enucleation such as photocoagulation, local resection and especially radiotherapy are applied in an increasing number of cases.

With regard to these "conservative" types of treatment, laser photocoagulation is currently not that much advocated, but is still used by some authors in selective cases of small uveal melanomas (Shields, 1991; Damato, 1993; Hungerford, 1993). Secondly, the surgical intervention can sometimes be confined to local resection of the tumor in particular cases of posterior choroidal melanomas. Because of the technical difficulties of this surgical procedure, local resection of these tumors is only performed in a few specialized centers (Damato, 1993; Shields, 1991). After local resection, especially, of a nasally located posterior choroidal tumor, a good vision of over 6/12 is retained in 57 % of the cases (Damato, 1993). In cases of uveal melanoma with massive orbital involvement, then, orbital exenteration is sometimes indicated for local tumor control. Since an exenteration will probably have no influence on the patient's survival, it can be disputed whether an orbital exenteration has to be performed in cases of episcleral extension that could also be removed by enucleation (Shields, 1993; Kersten, 1985; Shammas, 1977; personal data). Finally, in cases of small, dormant tumors (tumor height< 3 mm) - tumors that show no signs of growth in serial examinations by

ultrasonography and fluorescence angiography, - some authors advocate mere observation until tumor enlargement has been demonstrated (Butler, 1994; Augsburger,1993). However, a recent clinical study reports that 3 % of the patients with tumors less than 3 mm in height and up to 19% of them in case of proven tumor growth will develop distant metastases after all (Shields, 1995). Therefore, even in cases of apparently small dormant uveal melanomas, prompt treatment appears to be recommendable.

In most cases, however, the choice of treatment is one between enucleation and radiotherapy of the tumor-containing eye. For several ophthalmologists the principle choice has become radiotherapy, applied by means of a radioactive episcleral plaque with cobalt-60 (Stallard,1959), ruthenium-106 (Lommatzsch, 1973), or iodine-125 (Packer, 1980), or by irradiation with charged particles or proton beams (Gragoudas, 1980) or helium-ions (Hungerford, 1993; Shields, 1991; Char, 1980).
Yet, there is much discussion about specified indications and the choice of the optimal dose.

One of the major reasons to perform radiotherapy, or any conservative treatment, is to retain the patient's vision. Most authors advocating radiotherapy describe a useful vision as a vision of over 20/200. However, although they report a visual acuity of more than 20/200 in about 90% of the cases after 1 year, after two to three years this has decreased to 73% and 50%, respectively (Young,1994; Quivey, 1993; Guyer,1992; Shields,1991). Additionally, no differences have been found in vision-depending-activities between groups of patients either treated by enucleation or by radiotherapy; neither do patients encounter significant visual problems during their vision-depending-activities after enucleation of one eye (Augsburger, 1994; Edwards, 1991). In other words, the choice between enucleation and radiotherapy would not have to be hindered by arguments of respective bother to the patient. Although, a few comparative studies could not demonstrate a significant difference in survival between the two types of treatment (De Potter, 1994; Augsburger, 1990; Adams 1988), one should keep in mind that there are hardly any proper studies available with long-term follow-up data and a fixed protocol. Recently, the collaborative ocular melanoma study group (COMS) designed a multicentre trial on the topic of episcleral plaque therapy versus enucleation; it is expected that this COMS study will clarify some of the issues (Straatsma, 1988).

After any type of initial treatment, the patients have to be followed regularly. Patients treated by radiotherapy need a frequent ophthalmic check-up in the post-treatment period in consideration of direct complications and tumor control. Post-radiotherapy complications, such as neovascular glaucoma, scleral necrosis and tumor regrowth, lead to an indication for eventual enucleation in 6 to 23 % of the patients (Egan, 1989; Shields, 1990; De Potter, 1994). In case of enucleation, the follow-up protocol in our own clinic includes ophthalmoscopic examination of the non-affected eye, inspection of the socket, palpation of preauricular and submandibular lymph nodes, and liver-enzyme tests. With regard to the latter, in case of demonstrated changes in the liver-enzymes, a liver ultrasonography is performed in order to detect possible liver metastases (De Jong, 1987). These examinations are performed every three months in the first half a year, then every six months during the first two years, and thereafter annually (De Jong, 1987).

1.1.3 Metastatic disease and survival

Despite all effort that has been put in refining diagnosis and treatment of the primary tumor described in the preceding paragraphs, the life expectancy of uveal melanoma patients has not improved over the last decades. Still, 50 % of the patients sooner or later develops metastatic lesions that lead to a sure death. Survival rates, assessed by the actuarial survival estimate (Kaplan-Meier curves) or the multivariate analysis (Cox proportional hazard model), show survival percentages of 75 %, 65 % and 55 % respectively 5, 10 and 15 years after initial treatment (Luyten, 1995; Mclean, 1993; Gamel, 1993; Diener-West, 1992; Jensen, 1982). Self-evidently, these analyses are based on tumor-related deaths, while deaths by other causes simply count as the last date of follow-up of the patient in question (Seigel, 1990). By comparing the hypothetical survival rates of uveal melanoma-related death with the actual survival time, the asymptote of the hypothetical survival rates reaches a certain hypothetical value which is the function of the survival time of the uncured patients. The median survival time of 6.5 years that was mentioned above was assessed this way, in a large study of 4726 uveal melanoma patients that also demonstrated a cure rate of 42 % (McLean, 1993). In other words, 50 % of all patients who will die from metastatic uveal melanoma die within 6.5 years after initial treatment; the highest mortality rate is found

in the second to fourth year following initial treatment. Finally, a significant longer survival time has been found among younger patients, that is, patients below 50 years of age (Gamel,1993; Kath,1993; Gragoudas, 1991; Rajpal,1983), and compared to men, women have a longer survival time (Luyten, 1995; Folberg, 1993; Rajpal, 1983).

Uveal melanoma disseminates almost exclusively haematogenously with a strong preference for the liver. Liver involvement is found in 56 to 88 % of the patients as the first symptom of metastatic disease, while in the final stage the liver is involved in up to 95% of the cases. Other organs are much less frequently affected: the lung in 24-46%, the bones in 17-29%, and the skin in 15-34% of the cases (Kath, 1993; Albert, 1992; Gragoudas, 1991; Rajpal, 1983). After metastatic uveal melanoma is diagnosed the patient's life expectancy at the time of diagnosis is 2 to 7 months with, and 19 months without liver involvement (Kath, 1993; Albert, 1992; Gragoudas, 1991; Rajpal, 1983; Bedikian, 1981; Char, 1978). The orbital recurrence rate ranges from 0.5 to 3 %, but increases to as high as 23% in cases of extraocular extension of the primary uveal tumor. Death by an orbital recurrence itself is exceedingly rare; however, when a patient presents an orbital recurrence, his life expectancy is very poor (0 to 8 %), because he or she is almost sure to develop distant metastases (Kersten, 1985; Affeldt, 1980; Shammas, 1977). Since the eye itself lacks a lymphatic drainage system, lymphatic dissemination is only found as a late event in the metastatic process in cases of uveal melanoma with extraocular extension.

Considering the short survival time of uveal melanoma patients suffering from metastatic disease, especially when the liver is involved, we have to deduce that the success of treatment modalities is to date rather limited. For the treatment of metastatic uveal melanoma six approaches have been described: combined chemotherapy (Kath, 1993; Gragoudas, 1991; Einhorn, 1974), chemoimmunotherapy (Gragoudas, 1991; Bedikian, 1981), chemotherapy and surgery (Kath, 1993; Fournier, 1984; Rajpal, 1983), intrahepatic chemoembolization (Kath, 1993; Mavligit, 1988), adjuvant chemotherapy (Sellami, 1986) and immunotherapy (Mitchell, 1994). Some authors advocate the application of adjuvant therapies, such as immunotherapy with the methanol-extraction residue of the Calmette-Guerin bacille, or chemotherapy including vinblastine, thiotepa, methotrexate, deticene and procarbazine (Mclean, 1990; Sellami, 1986). However, neither the main, nor any of the adjuvant therapies

have proved truly effective yet. In only a few cases a partial response to treatment could be demonstrated and then only when chemotherapy was used, whether or not combined with an another treatment modality; one such study reports an extension of the mean survival time of 3 months (Gragoudas, 1991). Besides, one case report describes the remarkable successful treatment of a primary uveal melanoma by the sole application of active specific immunotherapy with cutaneous melanoma lysates (DETOX), that induced a reduction of the tumor height from 4.2 to 2.4 mm (Mitchell, 1994). A pilot study that investigates the effects of treatment of metastatic uveal melanoma by recombinant alpha-2b-interferon with dacarbazine, vincristine, bleomycin and lomustine has recently been initiated by the Ophthalmic Oncology Group of the EORTC (Pyrhönen, 1992).

1.2 Aims and scope of the thesis

In the preceding section of this chapter, the clinical aspects of uveal melanoma have been described; the present and next sections are dedicated to a presentation of the aims and background of the thesis itself, the collection of articles taken up in Chapters 2 to 9 that also have been published or submitted elsewhere in the period between 1993-1996. As will have become clear from section 1.1, less than 2 % of the uveal melanoma patients presents distant metastases detectable with the currently available methods at the time of diagnosis of the primary tumor, while 50 % of them eventually dies from it. The development of new anticancer strategies is therefore particularly significant for these patients.

The studies in this thesis ultimately aim at the development of a therapy adjuvant to the available primary treatment modalities, directed at counteracting any aspect of possible metastatic dissemination, as well as at a more effective therapy of metastatic uveal melanoma itself. Evidently, metastatic dissemination involves a complex series of genotypic and phenotypic changes; dissemination of uveal melanoma includes tumor cell motility, invasion of the tumor border, the sclera and the vascular system, survival in and extravasation from the circulatory system, colonization of a distant site, angiogenesis, and avoidance of host immune responses (Fidler, 1990). To date, the development of anti-metastatic therapeutic agents or, in general, antimetastatic modalities, has been hampered by incomplete knowledge of the metastatic process; apparently, quite a large number of genotypic and phenotypic changes have to be elucidated before new anticancer strategies can be practiced (Liotta, 1991). With the ultimate aim of developing new therapeutical modalities, this thesis intends to contribute to the knowledge of the nature and causes of metastatic uveal melanoma by investigating some of the key elements of these changes involved in the metastatic process.

In this context three (interrelated) questions arise: First of all, what are the origins of the metastatic seedings? How can we localize and eradicate these, preferably in the very first stage of development and before they become clinically manifest? Secondly, how do we identify which patients are especially at risk, i.e. what clinical and histopathological factors are involved in the metastatic process, so that we may adapt their treatment accordingly and as early as possible? Thirdly, how can uveal

melanoma-related metastatic disease be successfully treated? Might its development be blocked, for instance by the early application of immunotherapy?

Regarding the first question, the eradication of (micro-) metastases at the earliest stage, we studied the procedure of pre-enucleation irradiation (Chapter 2). Regarding the second question, we investigated the morphological and immunohistochemical properties of clinical manifest metastases and their corresponding primary uveal tumors (Chapter 3), as well as the possible role of neural cell adhesion molecules (Chapter 4) and of the putative metastasis suppressor gene *NM23* (Chapter 5). In view of the third question, the treatment of metastatic disease, we undertook several experimental studies. Firstly, we established and characterized primary and metastatic uveal melanoma cell lines, vital to the study of the biological properties of uveal melanomas (Chapter 6). We then studied the expression of melanoma-specific and associated antigens in uveal melanoma cell lines. These cell lines might be used in specific immunotherapy (Chapter 7). Furthermore, the actual susceptibility of uveal melanoma cells to natural killer cells was experimentally tested in nude mice (Chapter 8). Finally, we experimented with growth of uveal melanomas in a chicken embryo model (Chapter 9). The background of these studies will be introduced in more detail in the following section.

1.3 Introduction to the studies

1.3.1 The origins of metastases (Chapter 2)

Much discussion remains on the origins of the initial metastatic seedings that give rise to the distant metastatic lesions of uveal melanoma. Among others, Zimmerman and associates suggested that tumor cells are disseminated by the physical manipulation of the tumor-containing eye during enucleation (Zimmerman, 1978). In view of this possible risk, the procedure of pre-enucleation irradiation was introduced, presuming that eradication of these micro-metastases by irradiation would prevent the eventual development of distant metastases (Char, 1982). Yet, Manschot and Van Peperzeel (1980) argued that possible micro-metastatic seedings induced by enucleation would not become manifest within 6 years following this procedure, which would not explain the high mortality rate in the second to fourth year after the treatment (see section 1.1.3). They therefore suggested that the micro-metastatic spreading occurs even before, and independent of treatment (Manschot, 1980). To date, data on the subject have mainly been in favor of Manschot's hypothesis (Manschot, 1992, id, 1980). Firstly, since uveal melanoma metastases are rarely discovered prior to the primary tumor and they can occur many years after enucleation (see section 1.1.3), in survival rates the major function for the survival time is the growth rate of the cells, not the tumor size of the uveal melanoma. In other words, large tumors consist of more aggressively growing cells compared to small tumors. Secondly, the procedure of pre-enucleation irradiation, applied in the several centres in various doses, has not proved to have any beneficial effect on survival (Luyten, 1995; Augsburger, 1990; Bornfeld, 1989; Kreissig, 1989). On the contrary, in one study irradiation before enucleation showed an inverse effect on survival (Char, 1988). These findings suggest a minor role of the physical manipulation of the tumor-containing eye during enucleation. The cure rate and survival time calculations as described in section 1.1.3 are in concordance with the hypothesis of Manschot, therefore we can possibly adopt the view yet that metastatic uveal melanoma and melanoma-related death is the eventual consequence of micro-metastasis that takes place independent of the initial treatment. Chapter 2 of this thesis discusses a comparative clinical study of a group of uveal melanoma patients treated by preoperative irradiation and a historical control group treated by enucleation alone, which considered a considerably longer follow-up (71/2 years).

1.3.2 Clinical and histopathological factors (Chapter 3, 4 and 5)

Regarding the choice of how to manage patients with a primary uveal melanoma, the risk of developing metastases has to be inferred mainly from clinical observations. The determination of these clinical prognostic factors has become more important with the increase in assessment of radiotherapy and other conservative treatments (see 1.1.2), because histological material can not be obtained in these types of treatment. Several clinical and histopathological prognostic factors have yet been proposed; obviously, their clinical impact is of limited value as long as there are no treatment protocols directed by these clinical prognostic factors, nor successful adjuvant therapies for high-risk patients (Mitchell, 1994; Albert, 1992; Mclean, 1990; see also section 1.1.3).

From clinical observation the following three clinical risk factors have been elucidated: largest tumor dimension in contact with the sclera (LTD), location of the anterior tumor margin, and the presence of extraocular extension of the tumor (Shields, 1995; Rummelt, 1995; Augsburger, 1990). Furthermore, as mentioned in 1.1.3, patients of higher age and of the male sex have proved to have an increased risk to develop metastases (Rummelt, 1995; Luyten,1995; Folberg, 1993; Coleman, 1993). With respect to small uveal melanomas, several other clinical risk factors have been appointed, of which the most important one is demonstrated growth of the tumor. Growing small melanomas compared to non-growing result in an eight times increased risk to develop metastases (Shields, 1995). Besides demonstrated tumor growth, posterior tumor margin touching the optic disc and initial greater tumor thickness were found to be important predictive factors for metastasizing of small melanomas (Shields, 1995). Predictive factors for tumor growth itself are: initial tumor thickness, posterior tumor margin touching the optic disc, symptoms of flashes, floaters, and blurred vision, orange pigment on the surface of the tumor, and the presence of subretinal fluid (Shields, 1995).

In tumors of patients treated by enucleation, additional histopathological parameters can be evaluated, such as cell type, vascular networks, cytomorphometric assessments of the nucleoli and mitotic figures, as well as cytogenetics and tumor-infiltrating lymphocytes. Of these histopathological parameters, when evaluated along with the clinical prognostic factors for the risk of developing metastases, the assessment of the largest tumor diameter (LTD) is again the most reliable independent

one (Coleman, 1993; McLean, 1993; Diener-West, 1992). The use of the cell type classification as an independent prognostic factor is less powerful. Although the modified Callender classification is mostly applied (spindle, mixed and epitheloid), several studies have simplified this classification to the presence or absence of epitheloid cells (Mooy, 1995; McLean, 1983). To improve the prognostic value of the cell morphology, more reproducible parameters have been developed, as for instance mean diameter of the ten largest nucleoli (MLN) (McCurdy, 1991). The assessment of morphometric analysis is however time consuming and therefore not very feasible for daily practice (Mooy, 1995; Rummelt, 1995).

Again in the multivariate analyses of cure rates and survival time, LTD has been shown to provide the greatest prognostic insight (Gamel, 1993). A meta-analysis of 39 publications showed the following 5-year mortality rates - which are higher than is generally presumed - in relation to LTD: 16 % of the patients with a small melanoma (LTD < 10 mm LTD); 32 % of those with a medium melanoma (LTD 10 - 15 mm), and 53 % of those with a large melanoma (LTD > 15 mm) (Diener-West, 1992). Multivariate analyses further show that the percentage of mitotic figures as well as the occurrence of tumor-infiltrating lymphocytes (TIL) is significantly associated with uveal melanoma-related death (Whelchel, 1993; Folberg, 1993; De la Cruz, 1990). The prognostic value of these parameters and some other immunohistochemical markers for proliferative activity were found to associate with uveal melanoma-related death, such as the expression of MIB1, MYC (Mooy, 1995; id., 1995; id., 1990; Bardenstein, 1991), still have to be investigated in a prospective study (see section 1.3.3.2 below on cytogenetics).

Although LTD is the most frequently used prognostic factor, recent reports show that the presence of closed vascular loops or networks in uveal melanomas is highly associated with melanoma-related death, and that these vascular networks might be of even more prognostic significance than LTD (Rummelt, 1995; Pe'er, 1994; Rummelt, 1994; Folberg, 1993). Finally, in order to select patients at risk, a new minimal detection technique using reverse-transcriptase polymerase-chain-reaction (rt-PCR) has been developed. rt-PCR detects the tyrosinase gene and is thereby able to demonstrate disseminating melanoma cells in peripheral blood of patients suffering from both primary and metastatic disease (Tobal, 1993). The clinical value of this technique for the

detection of circulating melanoma cells is however not yet clear (Foss, 1995).

In Chapter 3, 4 and 5 three immunohistochemical studies that should contribute to the formulation of differences between primary and metastatic uveal melanoma are described. Chapter 3, besides a morphological study of primary and related metastatic lesions, presents an evaluation of the sensitivity of these lesions to three melanoma-associated antibodies: HMB-45, S100 and NKI-C3. Chapter 4 investigates, according to the growing evidence that cell adhesion molecules may influence the binding and invasiveness of human cancers (Niederkorn, 1993, Nicolson, 1988), whether there is an association between the percentage of neural cell adhesion molecules present in the tumor cells and their metastatic behavior. Chapter 5, then, following studies on breast cancer (Steeg, 1991), investigates whether an inverse relationship exists with regard to the expression of the *NM23* gene and uveal-melanoma related death. (For a detailed description of the cytogenetics involved in uveal melanoma, see section 1.3.3.2 below).

1.3.3 Experimental studies (Chapter 6 to 9)

1.3.3.1 Uveal melanoma cell lines (Chapter 6)

The study of biological and molecular properties of the successive steps involved in melanoma progression, for instance in an experimental animal model (see section 1.3.3.3 below and Chapters 8 and 9), requires the availability of cultured cell lines. To date, only a few continuously growing cell lines have been established of primary uveal melanomas, and of metastatic uveal melanomas none (Ma, 1995; De Waard-Siebinga, 1995; Massarelli, 1994; Aubert, 1993; Soulieres, 1991; Kan-Mitchell, 1989). In contrast, several cell lines have successfully been established of cutaneous melanomas. This contrast can possibly be explained by the relatively high prevalence of cutaneous melanoma compared to uveal melanoma (10:1) (Albert, 1984). Furthermore, cutaneous melanomas are more feasible to culture as they frequently metastasize to lymph nodes, where the lesions are easily obtained. Metastatic uveal melanoma material, on the other hand, is difficult to obtain because uveal melanomas lead mainly to hepatic metastases, which are mostly confirmed by an examination of FNAB rather than by a true biopsy. Additional biopsies or excisions for culturing purposes have to be taken together with the diagnostic biopsies, because if they are

merely taken for culturing purposes, patients often do not agree. Besides, these patients are often treated beyond the scope of the ophthalmologist , which forms another impediment to the obtainment of fresh metastatic uveal melanoma material.

Chapter 6 reports on the successful establishment and characterization of two primary and three metastatic uveal melanoma cell lines. Although a culturing technique using a fibroblast feeding layer has also been described (Albert, 1984), our protocol follows most others in the use of a culturing medium of 10% fetal calf serum with penicillin and streptomycin to select the most aggressive and proliferating cells.

1.3.3.2 Tumor genetics and immunology (Chapter 7)

Tumorgenetics

In recent years several genes, both oncogenes and tumorsuppressor genes are found to play an important role in carcinogenesis and tumor progression. Mutations or loss of these genes leads to a growth advantage of the cell or in case of a metastatic process, outgrowth of a tumor cell population. Although some of these mutations in oncogenes or tumor suppresser genes can be used for diagnostic purposes, the underlying basis of genomic instability, leading to these mutations in the tumor cell is at largely unknown. Obvious candidate genes are genes involved in cell cycle control or genes involved in DNA repair mechanisms. An example of a gene involved in cell cycle control is the *CDKN2* gene which is mutated or deleted in familiar melanoma kindred's and several other human cancers (Cairns, 1995). This gene will be discussed in next paragraph. A second type of genes are involved in mechanism which lead to frequent mutations, deletions and insertions, and cloning of a human mismatch repair gene, *hMSH2*, linked to human non-polyposis colorectal carcinoma (HNPCC) was major step forward. To date several defects in other DNA repair genes are found in HNPCC *(hMSH2, hMLH1, hPMS2* and *hPMS1)* and are associated with genomic instability (Liu, 1996).

It is unknown whether in human uveal melanoma a similar mechanism leads to genomic instability and provide genetic changes whereby certain cells acquire new phenotypic traits that results in carcinogenesis, tumor outgrowth and further the metastatic process. In order to identify the genes and their proteins involved in carcinogenesis or tumor progression several approaches can be used.

Linkage studies

Familial uveal melanoma is quite rare (Young, 1994; Singh, 1994; Canning, 1988). In one report, the mean age of the patients with familial uveal melanoma at the time of presentation is significantly younger than usual (Canning, 1988). Affected members are always from one or two generations; the pattern of inheritance is suggested to be autosomal dominant with partial penetrance, or more polygenic. In a current study on familial uveal melanoma at our institute, three families were found with two affected members each, linked several generations from each other. In contrast, familial cutaneous melanoma is quite common: in about 10 % of the cases, at least one of the family members of the cutaneous melanoma patient is affected too.

Bergman and associates (1986) describe several families with the so-called familiar atypical multiple mole and malignant melanoma syndrome (FAMMM), which has been mapped on chromosome 9p21 (Cannon-Albright, 1992; Gruis, 1993). Patients from FAMMM families develop multiple naevi, atypical naevi (dysplastic naevi) and cutaneous melanoma. Although several authors have described FAMMM patients having an ocular melanoma as well, there is still some controversy regarding the association between FAMMM and ocular melanoma (Seregard, 1995; Bataille, 1993; Vink, 1990; Taylor, 1984). The putative FAMMM gene, *CDKN2,* or multiple tumor suppressor 1 *(MTS1)* gene, that has been cloned, encodes for the protein $P16^{INK4}$, which was previously identified through its ability to inhibit cyclin-dependent kinase-4 (CDK-4) (Cairns, 1995; Kamb, 1994). Currently, our department of Ophthalmology in cooperation with the (MGC) Institute of genetics, Erasmus University, Rotterdam and the Department of Antropogenetics and Department of Dermatology, Leiden University, The Netherlands, investigates the occurence of *CDKN2* gene mutations in uveal melanoma in vivo and in uveal melanoma cell lines by use of the single-strand-conformation polymorphism (SSCP) technique.

Cytogenetics

Cytogenetic analysis of tumors may provide information on nonrandom chromosomal aberrations like deletions, translocations and amplifications, for it provides the possibility of detailed studies of loss or gain of certain oncogenes and tumor

suppressor genes located on the chromosomes of interest. Cytogenetic analyses of uveal melanoma have been performed on limited series of primary uveal melanomas, but sofar not on metastatic uveal melanomas. The most common genetical changes that were found in primary uveal melanoma are: loss of chromosome 3, 6q and 8p, and gain of chromosome 6p and 8q (Horsthemke, 1992; Horsman and White, 1993; Wiltshire, 1993; Dahlenfors, 1993; Magauran, 1994; Singh, 1994). Monosomy of chromosome 3 and isochromosome 8q have been found to be associated with ciliary body melanomas and high risk for metastatic disease (Prescher, 1992; Sisley, 1990). Two studies on uveal melanoma that used a recently developed technique, comparative genomic hybridization (CGH) (Kallioniemi, 1992) have confirmed these findings (Speicher, 1994; Gordon, 1994). Hence, loss of chromosome 3 in cells of the primary tumor might be used as a prognostic factor.

The role of known oncogenes and tumor suppressor genes that have previously been described with regard to other types of cancer has been studied with respect to uveal melanoma too. Firstly, whereas in cutaneous melanoma activating *NRAS* mutations that associated with both sun-exposure and malignant progression were frequently found (Ball, 1994), two studies of uveal melanoma could not demonstrate *NRAS, HRAS* or *KRAS* mutations (Soparker, 1993; Mooy, 1991). Secondly, the tumor suppressor *TP53* gene, located on chromosome 17p and encoding a M_r 53 Kd nuclear phosphoprotein, is known to suppress cellular proliferation in its native form, whereas the mutant form controls oncogenic potential (Harris, 1993; Hollstein, 1991). In one study of primary uveal melanoma, an overexpression of the TP53-protein was demonstrated in 12 out of 18 melanomas; two of them showed a point mutation in TP53 (Tobal, 1992). Furthermore, a Victorian family has been described in which four generations developed uveal melanomas together with breast cancer. In this family, immunohistochemical analysis showed a mutant TP53 in the tumors. Unfortunately, germ line material was not tested (Jay, 1993). Thirdly, the protein product of the *MYC* oncogene is known to play an important part in cellular proliferation, differentiation, and tumor formation, presumably by modulating the expression of genes involved in these processes (Peltenburg, 1994). Prognosis-related enhanced expression of this gene has been observed in human carcinomas (Peris, 1991). In cutaneous melanoma, high MYC expression inhibits the expression of class I HLA, which makes the cells

less prone to MHC-restricted cytotoxic T-lymphocytes (CTL) immunity on the one hand, and more sensitive to natural killer (NK) cells on the other (Versteeg, 1988). We will come back to this below. The *MYC* gene is located on chromosome 8q24 (Human gene mapping 11, 1991), which shows a high expression in human uveal melanoma (Speicher, 1994). In uveal melanomas, the immunohistochemical staining of the MYC protein was found to correlate with a proliferative index assessed by flow cytometry (Royds, 1992). Another immunohistochemical study shows an inverse correlation between the MYC and the BCL2 protein, which plays a role in apoptosis. Furthermore, the expression of MYC is significantly associated with uveal-melanoma-related death (Mooy, 1995).

Finally, the *NM23* gene, due to its differential expression on related low and high metastatic potential murine K-1735 melanoma cell lines, is proposed as a metastatic suppressor gene (Steeg, 1988). To date, two human *NM23* genes have been identified (Stahl, 1991; Rosengard, 1989), each encoding for a 17 kDa protein; they have been mapped on chromosome 17q21.3-22 (Backer,1993). Expression of *NM23* in human tumors is most extensively studied in breast cancer. Reduced mRNA and protein levels in ductal breast cancer have proved to be correlated with lymph node metastases, decreased disease-free survival and decreased overall survival (Royds, 1994; id, 1993; Hennessy, 1991; Barnes, 1991; Bevilacqua, 1989). A similar inverse relation between the expression of the *NM23* gene and reduced metastatic potential could be demonstrated for several other types of human cancer (Bertheau, 1994; Kodera, 1993; Yamaguchi, 1993; Florenes, 1992; Nakayama, 1992). However, in some types of cancer, *NM23* expression appears not indicative or, in reverse, is increased in highly metastatic tumors (Fishman, 1994; Leone, 1993; Engel 1993; Radinsky, 1992; Haut, 1991).

The role and prognostic value of NM23 protein expression in uveal melanoma is more elaborately described in Chapter 5 of this thesis; Cytogenetic analysis of primary and metastatic uveal melanoma cell lines is presented in Chapter 6. Cytogenetic analysis by karyotyping and fluoresence in situ hybridization (FISH) on fresh primary and metastatic uveal melanoma are currently performed at Department of Genetics of the Erasmus University Rotterdam.

Immunology

The major role of the immune system in controlling uveal melanoma has not yet been elucidated. This role can be deduced from clinical observations: firstly, primary uveal melanomas sometimes remain localized for long periods without showing any progression, and metastases are often only observed decades after enucleation of the primary tumor. Secondly, older patients having a primary uveal melanoma have an increased risk to develop metastases and when they develop metastatic disease, they have a significantly shorter survival time (see 1.1). Thirdly, although only in rare cases, spontaneous regression of primary and metastatic lesions has been described (Jensen, 1974).

Furthermore, the immunogenicity of uveal melanomas and their related metastatic tumors has been associated with the presence of lymphocytic infiltration. In 5 to 12 % of the primary tumors T-lymphocytes (CD3-positive) and to a lesser extent, numbers of natural killer (NK) cells, monocytes and macrophages can be observed. The lymphocytic infiltration comprises cytotoxic/suppressor (CD8-positive) and helper/inducer (CD4-positive) cells (Tobal, 1993; Meecham, 1992; Durie, 1990). The presence of lymphocytic infiltration has been associated with a better prognosis in cutaneous melanoma, but, curiously enough, with a poor prognosis in uveal melanoma (Whelchel, 1993; Folberg, 1993; De la Cruz, 1990). Most likely, this negative association with regard to uveal melanoma can be explained by the fact that the eye is an immune-privileged site and that, for the generation of a T-lymphocyte mediated immune response, disseminated tumors cells are necessary (Whelchel, 1993).

However, the fact that detectable quantities of lymphocytes can be found in uveal melanomas means that the immune system is somehow stimulated and hence, that these cells must express certain antigens that are recognized as foreign by the tumor-bearing host's immune system. It is commonly assumed that tumor cells, as a result of the expression of mutated or viral genes or a deregulated expression of normal genes, produce certain proteins that are either not produced at all or in much lower quantities in normal cells (Germain, 1994). These abnormal proteins become tumor antigens at the cell surface that serve as target for effectors of natural immunity, such as NK-cells, or for specific immune responses by cytotoxic T-Lymphocytes (CTL's), if they are associated to major histocompatibility complex (MHC) class I

molecules and ß2 microglobulin (Germain, 1994). Once these surface antigens or peptide/MHC class I molecule complexes are recognized by NK cells or specific CTL's, these kill the infected or the tumor cell. The immune system thus provides a useful target for cancer treatment modalities.

The tumor antigens expressed by melanoma cells can either be melanoma-specific, that is unique to melanomas, or melanoma-associated, in which case they are not only present in melanoma cells but in all melanocytic derived cells. The first discovered melanoma-specific antigen, MAGE1 (melanoma antigen), was elucidated by CTL clones isolated from cutaneous melanoma patients and cloned by use of peripheral blood lymphocytes (PBL's) of a patient who had been immunized with autologous tumor cells (Van Der Bruggen, 1991; Herin, 1987). The immune system of the patient recognized the protein encoded by this gene in association with HLA-A1 (Traversari, 1992). The CTL clones were subsequently used to select antigen-loss variants and this led to the definition of several other antigens that are expressed by autologous melanoma cells (Van De Eynde, 1989). Thus the *MAGE1* gene, that lacks similarity to other sequences in current databases, was found to be a member of a multigene family. The product of another member of this family, called *MAGE3*, has also been shown to be recognized by HLA-A1-restricted T-cells (Gaugler, 1994). *MAGE1* and *MAGE3* genes are expressed in cutaneous melanomas in a frequency of respectively about 40 % and 69 %. They have been found in several other types of tumors as well (e.g. squamous cell carcinoma, small cell lung carcinoma and sarcomas) (De Smet, 1994), but not in normal tissue except for the testis (De Smet, 1994).

Two melanoma-associated antigens, that besides in tumor cells, are expressed in normal melanocytes as well, are *tyrosinase* and *GP100*. Both genes are involved in the melanin synthesis and are also recognized by CTL's. The *tyrosinase* gene was cloned by means of CTL clones which were obtained from PBL's of cutaneous melanoma patients after repeated in vitro tumor stimulation (Wölfel, 1994; Brichard, 1993); its protein product was found to be recognized at the cell surface in association with the HLA-A2 molecule. The product of the other melanoma-associated antigen *GP100* is recognized by HLA-A2-restricted tumor infiltrating lymphocytes (TIL's) as well (Bakker, 1994; Kawakami, 1994a; Adema, 1994; id, 1993;). The antigen encoded

by *GP100* cDNA is recognized by the monoclonal antibodies NKI-beteb, HMB-50 and HMB-45 (Adema, 1993); these melanocytic lineage-specific Mabs are among the best diagnostic markers for cutaneous and uveal melanoma available to date (Steuhl, 1993; Vennegoor, 1988; Vogel, 1988; Van Der Pol, 1987; Gown, 1986). Recently, a third melanoma-associated antigen has been cloned, *MART1*, which has highly immunogenic common epitopes that are recognized by the majority of HLA-A2-restricted melanoma specific TIL's isolated from HLA-A2 melanoma patients (Kawakami, 1994b; id, 1994c).

Besides expression of antigens at the cellular membrane, recognition and cytolytic response by CTL's requires expression of MHC molecules. Cells in which the MHC expression is down-regulated are not recognized by CTL's. In 75 to 85% of uveal melanomas, MHC class I molecules are expressed (Meecham, 1992). Endocytosed antigens and exogenous antigens are associated with MHC class II molecules. However, recently it has been shown that these endocytosed (exogenous) antigens can also be presented in the context of MHC class I molecules (Kovacsovics, 1995). MHC class I expression in uveal melanoma metastases has not been reported on yet. In other types of cancer no correlation exists between the level of MHC class I expression and immunogenicity. It appears likely that a certain MHC molecule has a strong preference to bind a particular peptide, and that the immunodominant peptide from a certain antigen binds only to a particular MHC class I molecule; hence, only tumors that express the allele of that peptide will be immunogenic (Germain, 1994). Other MHC class I molecules will probably also bind this peptide, but much less effective.

As mentioned above, the immune system appears as a promising target, or tool, in the treatment of melanoma. From clinical and experimental studies on cutaneous melanoma, evidence was obtained that specific CTL's indeed play an important role in the antitumor response against the melanoma (Barth, 1990; Mule, 1987; Greenberg, 1981). In vitro studies with TIL's and PBL's isolated from cutaneous melanoma patients have demonstrated that these CTL's are able to recognize melanomas in a MHC-restricted manner, indicating that CTL's recognize specific tumor antigens.(Coulie, 1992; Topalian, 1989; Herin, 1987; Knuth, 1984; Mukherji, 1983). In a variety of murine tumor models, the adoptive transfer of murine TIL's has

shown these to mediate in the regression of established metastases (Spiess, 1987; Rosenberg, 1986). TIL's can also mediate in cancer regression when adoptively transferred to the autologous patient (Rosenberg, 1992; id, 1988).

In vitro studies of uveal melanoma also demonstrated melanoma-specific CTL reactivity in a TIL culture. The TIL culture was expanded by IL-2 and the lymphocytes were found to destroy the patient's own uveal melanoma cells and not those derived from other patients, thus showing a specific immunoreactivity for the autologous tumor cells (Ksander, 1991). Autologous as well as allogenic cell lines have been used to generate CTL clones from PBL's by mixed lymphocyte/tumor cultures (MLTC). A higher amount of melanoma-specific CTL clones could be derived from autologous compared to allogenic MLTC's (42% versus 14%) (Huang, 1994; Kan-Mitchell, 1991). Since it is relatively difficult to culture uveal melanoma cells, and melanoma-specific CTL can also be isolated from allogenic MLTC's, active specific immunotherapy of uveal melanoma patients should be feasible.

In addition to CTL's, natural killer (NK) cells are probably also effector cells of natural and acquired immune responses to melanomas. NK's constitute a heterogeneous group of cells expressing CD16 that can lyse both autologous as well as allogenic tumor cells (Warren, 1993). The basis for their specificity is not yet understood. Unlike T-cells, NK-cells do not express T-cell antigen receptors, and they kill target cells in a non major histocompatibility complex (non-MHC) restricted way. Low MHC class I expression in tumor cells makes the target cells more susceptible to NK's (Ma, 1995; Versteeg, 1989; id, 1988). The enhancement of NK-cells by alpha-interferon or Linomide in a murine ocular melanoma model demonstrated a significant decrease in pulmonary metastases. This shows that natural killer cells, like CTL's, are probably important primary effector cells for the control of metastasis, which might be utilized in treatment modalities of metastatic disease (Harning, 1989; Yokoyama, 1986).

The expression of the melanoma-specific *MAGE*-family genes as well as the two melanoma-associated genes, *tyrosinase* and GP100 were evaluated in uveal melanoma, using the cell lines that had been established in the institute (see 1.3.3.1). In order to find out whether uveal melanoma cells constitute an effective target for specific immunotherapy, the MHC Class I expression in the cell lines was also studied. This experiment is discussed in Chapter 7.

1.3.3.3 Animal models (Chapter 8 and 9)

The study of growth and metastatic capacity in an experimental animal model is one of the main interests of the establishment of human uveal melanoma cell lines (see 1.3.3.1). The major problem with animal models is the host's immune response against the donor material. Furthermore, to be able to compare the metastatic process in the model with the human situation, not only human uveal melanoma cell lines are necessary but also the local organ-specific factors are important; to study growth, invasion and dissemination. Therefore orthotopic transplantation is advocated (Ma, 1995; Niederkorn, 1993; id, 1981).

To date, the best experimental model is the nude mouse. The athymic, immunodeficient nude mouse provides an in vivo model with low residual immunity (Hill, 1991). Actually, it did not appear very successful for human uveal melanoma initially, but the problems turned out to be due to the use of uncultured melanoma cells that were transplanted heterotopically in the subcutis (Albert, 1980). Furthermore, it has become increasingly clear that tumors have to be transplanted at an orthotopic site to form metastases. Two other research groups did succeed in an orthotopic transplantation of human uveal melanoma cells that demonstrated metastatic spreading (Ma, 1995; Niederkorn 1993; Soulieres, 1991). A comparable murine model has been used to study the effect of difluoromeththylornithine and dacarbazine (DTIC) in preventing metastatic spreading of B16 melanoma (murine, cutanous) cells that had been injected subcutaneously and intracamerally (Sanborn 1992; id, 1992). This well-characterized animal model make use of B16 melanomas cells, which is a murine cutaneous melanomas transplanted in the syngenic C57BL/6 mice. These B16 melanomas metastasize to the lungs as all cutaneous melanomas (Harning 1987; id, 1989; Niederkorn JY 1984; id, 1984; id, 1984). This model is however not applicable for human uveal melanoma cells.

Two other mouse models that should be quite appropriate for experimental studies are the severely immunocompromised nude mouse (CB-17 SCID) and the triple-immune-deficient beige mouse (bg)/nude (nu)/xid (BNX). Both species lack functional B- and T-cells, while in the nude mice only the T-cells are lacking (Hill, 1991; Mulé, 1991). Furthermore, the BNX mouse does not possess functional lymphokine activating killer (LAK) cells. However, these models have not yet been

applied in research on human uveal melanoma.

Another animal model to study growth of human uveal melanoma cells is the rabbit immunosuppressed by cyclosporin. In one study, an OCM-1 human uveal melanoma cell line derived from a primary uveal melanoma was xenografted in the anterior chamber of the rabbit. Growth could be demonstrated, but no metastatic seeding was obtained (Kan-Mitchell, 1989). The main problem of this model is that the immunosuppressing cyclosporin can only be supplied for about 60 days because of its toxic effects on the rabbit, which seemingly limits its practical use in studies on metastasis and tumor biology. For the implantation of the so-called Greene melanoma, which is a murine cutaneous melanoma, the albino and Dutch rabbit have also been used. This model is applied in the study of new treatment modalities of the primary tumor but cannot be used to study tumor biology, as blood-born distant metastases have sofar not been obtained, xenografts express alien histocompatibility complex antigens and a C-type retro-virus is capable of transforming juxtaposed cells (Römer, 1992).

Iwamoto and associates (1991) developed a transgenic mouse with *Ret* oncogene expression under the control of a metallothionein promotor. Some of these transgenic mouse strains develop ocular melanosis and uveal melanoma. This is a novel mammalian model in which melanosis and melanotic tumors develop stepwise, triggered by a single gene; therefore, it has the advantage of providing the possibility to study the effect of one oncogene on the development of melanoma. Another transgenic mouse model (TySv40) has been developed bearing the *Sv40* oncogene under the control of the mouse tyrosinase promoter. These mice develop intraocular tumors that bear characteristics of retinal pigment epithelial cells and choroidal melanoma cells. The great advantage of the last mentioned model for the study of metastatic uveal melanoma is that haematogenously disseminated hepatic metastases do occur in this model (Anand, 1994).

In Chapter 7, as was indicated in the previous section, the expression of melanoma-specific and associated antigens in uveal melanoma cells is discussed as well as their MHC Class I expression. In sequel of this study, Chapter 8 discusses an experiment with a murine model in which the susceptibility of uveal melanoma cells to NK's was determined (see 1.3.3.2). Moreover, the author and associates succeeded

in developing yet another animal model for the study of uveal melanoma, namely a chicken embryo model. This experiment, that could provide new possibilities because of the immature immune system that chicken embryos possess, will be presented in Chapter 9.

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CHAPTER 2

NO DEMONSTRATED EFFECT OF PRE-ENUCLEATION IRRADIATION ON SURVIVAL OF PATIENTS WITH UVEAL MELANOMA

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ABSTRACT

Purpose: To evaluate the hypothetical effect of pre-enucleation irradiation on survival of patients with uveal melanoma.

Methods: In a prospective study between 1978 and 1990, 145 uveal melanoma patients were treated by irradiation in two fractions of 4Gy before enucleation. A historical control group of 89 patients with uveal melanoma treated by enucleation alone was operated on between 1971 and 1990. Patients were followed up until December 1992 or until death. The mean follow-up period was 65 months in the irradiated group and 88 months in the control group.

Results: The preoperatively irradiated group of patients showed no significant improvement of the survival rate after 71/2 years (75.9%) compared with the control group (72.1%). Preoperative irradiation was not associated with survival (P = .93), as assessed by Cox proportional hazard analysis, adjusted for age, gender, tumor location, tumor size, cell type, and year of enucleation. Women in both the irradiated and control groups had a better prognosis than men (P = .002).

Conclusions: Preoperative irradiation in this nonrandomized study had no effect on survival of patients with uveal melanoma.

INTRODUCTION

Whether micrometastasis can be induced by physical manipulation of an eye with uveal melanoma during the enucleation procedure, resulting in increased risk for tumor-related death, remains to be determined.[1,2] In animal models, melanoma cells can get into the circulation by manipulation of the tumor-containing eye.[3] To reduce this hypothetical effect of the surgical procedure on the survival of uveal melanoma patients, preoperative radiotherapy has been introduced.[2,4-7] Irradiation of the tumor before surgery might alter the tumor cells, thus preventing the implantation and growth of the circulating tumor cells after the enucleation procedure.[8-11] In the reported clinical studies no beneficial effect of pre-enucleation radiotherapy has been demonstrated.[4,12-14] Char and

associates[4] studied a small series of nonrandomized patients and found that preoperative irradiation with 20 Gy had a worse prognosis compared with patients treated by enucleation alone.

The purpose of our study was to evaluate the long-term effect of low-dose pre-enucleation irradiation on the survival of a large nonselected series of patients with uveal melanoma compared with a control group of patients treated by enucleation alone. To date this study is the largest series of nonselected uveal melanoma patients, treated preoperatively with a similar and relatively low dose irradiation, with the longest follow-up.

PATIENTS AND METHODS

Between 1978 and 1990 145 patients scheduled to have an eye enucleated for a choroidal or ciliary body melanoma were irradiated 48 and 24 hours before the operation with two fractions of 4 Gy electron beams (16 MeV) by means of a 5 x 5-cm anterior field on a linear accelerator. Between 1978 and 1982 patients were not treated at random either by pre-enucleation irradiation or by enucleation alone but were treated depending on the personal preference of the individual ophthalmologist in the institute, without selection otherwise. From 1982 on a standard protocol was used whereby all patients received preoperative irradiation unless there were reasons not to apply preoperative irradiation, such as acute angle closure glaucoma (one case), unexpected melanoma in a phthisic eye (one case), and a period of breakdown of the irradiation equipment (five cases). All patients treated by enucleation alone between 1971 and 1990 were used without selection as a historical control group (N = 89). From 1971 until 1985, postoperative irradiation was performed on the sockets of 15 patients who showed, on histologic analysis, extrascleral tumor growth. After 1985, postoperative radiotherapy was discontinued because of the lack of effect on survival. A few patients, with small to medium-sized melanomas not adjacent to the optic disk or macula and more than 60 years of age, were referred elsewhere for ruthenium plaque or proton beam irradiation from 1978 on. These cases were not included in this study.

All patients had a complete physical examination before surgery, including chest x-ray, and liver function tests; from 1978 on liver ultrasonography was routinely added.

No clinical evidence for metastatic disease was detected at the time of treatment. Patients were followed up twice a year in the first two postoperative years; after that time, annually. Follow-up examination consisted of ophthalmoscopy of the remaining eye, inspection of the socket, palpation of preauricular and submandibular lymph nodes, and liver-enzyme tests. Follow-up data on patients failing their appointment were obtained by annually contacting the general practitioner, local ophthalmologist, or both. To verify the date and cause of death, the files from the general practioner, hospital, or both were recovered. Uveal-melanoma-related death was diagnosed by proof from biopsy material or by strong clinical evidence (laboratory and radiodiagnostic) of metastatic disease. Otherwise the patients were considered to have died from other causes. Patients were followed up until death or until December 1992. In the pre-enucleation irradiation group there was no loss to follow-up; four patients were lost to follow-up in the control group. The total mean follow-up period was 65 months in the irradiation group and 88 months in the control group.

For each patient the following information was obtained: age of the patient at time of enucleation, gender, location of the tumor (ciliary body, posterior), cell type (spindle, mixed, epithelioid)[15], tumor size classified by tumor diameter, prominence, and extrascleral growth,[16] year of enucleation, follow-up time, and eventual cause of death. The cause of death was divided into two categories, uveal melanoma-related death and death by other causes. Classification of tumor size was as follows: T1, diameter of 10 mm or less and prominence of 3 mm or less; T2, diameter of 15 mm or less and prominence of 5 mm or less; T3, diameter greater than 15 mm and prominence greater than 5 mm; and T4, extrascleral growth.

All histologic data were reviewed by an ophthalmic pathologist (C.M.M.). From each tumor at least ten consecutive slides were examined.

The χ^2 test was used to compare the irradiated group with the control group for gender and tumor locations, the χ^2trend test for tumor size and cell type, and the Student *t*-test for age. Statistical analysis to study the effect of preoperative irradiation on survival was performed using Kaplan-Meier survival curves[17] and Cox proportional hazard analysis.[18] In both methods, survival was analyzed by using the uveal melanoma-related death. Death by other causes was censored and thus not used in the survival analysis as death but as last date of follow-up.

RESULTS

Five patients of the irradiated group and ten of the control group received postoperative irradiation because of episcleral tumor growth. The data on gender, mean age, tumor size classification,[16] cell type, and tumor location for the irradiated group and control group are shown in TABLE 1. For age and tumor size, the two groups differed significantly. The control group contained relatively older patients and had

TABLE 1
DISTRIBUTION OF PATIENTS AND VARIABLES BETWEEN GROUPS

	ENUCLEATION ALONE (N = 89)	PRE-ENUCLEATION IRRADIATION (N = 145)	P VALUE
1971 - 1977	54	0	
1978 - 1981	28	21	
1982 - 1990	7	124	
Average age	61.8	57.3	.03*
Gender			.91†
Male	51	82	
Female	38	63	
Location			.99†
Ciliary body	11	18	
Posterior	78	127	
Tumor size			.04‡
T1	8	14	
T2	11	37	
T3	38	62	
T4	29	32	
Cell type			.99‡
Spindle	44	75	
Mixed	37	52	
Epithelioid	7	18	
Necrotic	1	0	

* t-test
† chi-square test
‡ chi-square trend test (Tumor size of the preoperative irradiation group is relatively smaller compared to the enucleation group)

relatively larger (T4) tumors with extrascleral tumor growth. After 71/2 years of follow-up the survival rates for the preoperatively irradiated group and the control group were respectively 75.9% (95% confidence interval, 66.7% to 82.9%) and 72.1% (95% confidence interval, 60.0% to 81.0%). No significant difference in survival was found with the log-rank test (P = .26). According to Kaplan-Meier, the survival estimate curve of the preoperatively irradiated group and the control group is shown in FIGURE. 1. Both in the irradiated and in the control group (N = 234), women had a significant better survival than men (P = .03) (FIGURE. 2).

The Cox proportional hazard analysis (TABLE 2) was used to study the effect of preoperative irradiation on the survival of uveal melanoma patients, adjusted for prognostic covariates. The covariates used were irradiation, age, gender, tumor location, tumor size, cell type, and year of enucleation. There was no significant association with pre-enucleation radiotherapy (P = .93). The estimated adjusted hazard ratio (pre-enucleation radiotherapy vs enucleation alone) was 0.96 (95% confidence interval, 0.42 to 2.19).

TABLE 2

COX PROPORTIONAL HAZARD ANALYSIS

	BETA COEFFICIENT*	STANDARD ERROR	P VALUE	HAZARD RATIO†
Irradiation vs enucleation alone	-0.04	0.42	0.93	0.96
Age (yrs)	0.05	0.01	<0.001	1.06
Gender (Women vs men)	-1.0	0.32	0.002	0.37
Tumor location (anterior vs posterior)	0.19	0.40	0.64	1.21
Tumor size	0.48	0.18	0.008	1.62
Cell type	0.36	0.17	0.04	1.43
Year of treatment	0.06	0.259	0.818	1.06

* The Beta-coefficient is the natural logarithm of the hazard ratio.

† A hazard ratio, for instance 0.37 for sex, means that at each time during follow-up women have a chance of dying within one month equal to 0.37 times the chance of dying within one month for men.

Uveal melanoma-realted death was associated with older age (P < .001), male gender (P = .002), larger tumor size (P = .008) and epithelioid cell type (P = .04). No association was found with the year of treatment (P = .82). Postoperative radiotherapy on the socket showed no significant effect on survival (P = .44).

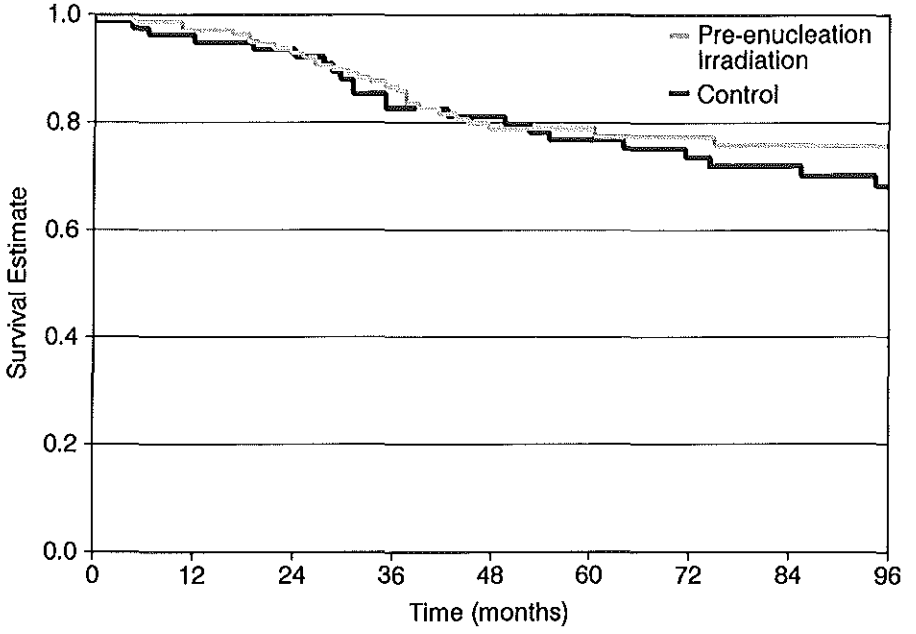

FIGURE. 1 Kaplan-Meier survival curves of the pre-enucleation irradiated group and the control group (P = .26).

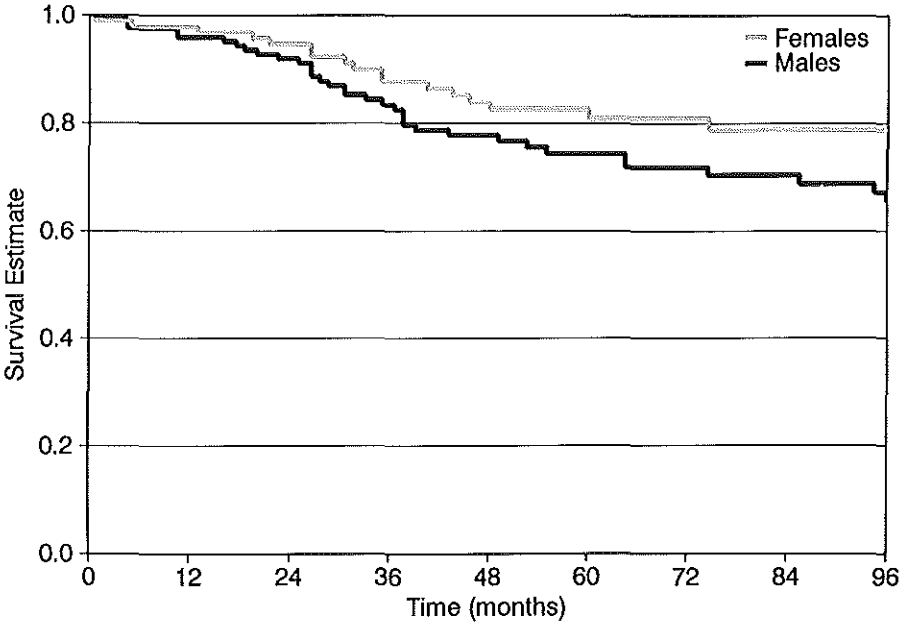

FIGURE. 2 Kaplan-Meier survival curves for women and men in the total group of 234 patients (P = .03).

DISCUSSION

In this study we found no long-term difference in survival of uveal melanoma patients treated by two fractions of 4 Gy before enucleation compared to patients treated by enucleation alone. In vitro and experimental studies have shown that an irradiation dose of 8 Gy in two fractions would be optimal.[8] Reduction of 90% and 95% of proliferation activity of melanoma cells after 8 and 20 Gy irradiation dose was demonstrated, respectively, by an in vitro study.[8] For human uveal melanomas, a significant reduction of the proliferation activity/mitotic rate was found after preoperative irradiation with 8[11] and 20 Gy.[19,20] Because of the experimental limits of these studies, no substantial difference between 8 Gy and 20 Gy pre-enucleation irradiation on the survival can be expected.

As a result of micrometastatic tumor spreading before or possibly during enucleation, about 50% of the uveal melanoma patients die within 15 years after initial treatment.[21-23] In our study the survival rate in the irradiated group was 75.9% after 71/2 years. After 15 years the survival rate decreased to 50%, but the number of patients was too small to give reliable rates.

In the Cox proportional hazard analysis, uveal melanoma-related death was associated with age, tumor size, and cell type, as described by other authors.[21-26] Interestingly, we found a significant difference in survival between men and women. Compared to men, women had a more favorable prognostic outcome. This difference in prognosis remained after adjustment for irradiation, age, tumor size, tumor location, cell type, and year of treatment. In several uveal melanoma studies on prognostic factors no significant difference was found between women and men.[21-24,26-28] In a discussion on survival after posterior uveal melanoma, Augsburger mentioned a better prognosis in women compared to men in an additional discussion of the study by Egan and associates.[28] More recently, this more favorable survival for women was confirmed by Folberg and associates.[25] Although studies on primary cutaneous melanoma are somewhat contradictory, a long-term survival study of primary cutaneous melanoma patients also showed that women have a better survival rate compared to men.[29,30]

The relative risk for development of metastases in patients treated by pre-enucleation irradiation has been reported to be fivefold greater compared to nonirradiated control patients.[4] In this study by Char and associates,[4] 41 patients were treated with 20 Gy (five fractions of 4 Gy) before enucleation. Patients with tumors larger than 15 mm in diameter or more than 5 mm elevation without evidence of extraocular extension (T3 tumors) were selected for irradiation. The unequal tumor size in both groups may be the cause of the observed effect. It is known that two fractions of 4 Gy irradiation on the two days prior to enucleation decreases the HLA class II antigen expression.[31] Perhaps a higher irradiation dose has a negative influence on the balance between tumoricidal irradiation dose and the possibility for the immune system to recognize tumor cells.

In another study,[12] 29 patients were treated by pre-enucleation irradiation and compared with a matched group of patients, who were treated by enucleation alone. The tumors were large (predominantly T3 tumors) and the median follow-up period for the surviving patients was 4.1 year in the irradiated group and 3.8 year in the enucleation group. The patients from the irradiated group received a total radiation dose of 20 Gy in five fractions. The five year Kaplan-Meier survival curves in both groups was not significantly different ($P > .5$). The cumulative 5-year survival probability was 63.9% for the 29 patients in the pre-enucleation irradiation group and was 57.9% for the control group. It could be interesting to determine whether the relative survival in these matched groups will remain equal over a longer time span, for instance, ten to 15 years.

In two other studies, the authors concluded that pre-enucleation irradiation produced no beneficial effect on survival; however, the radiation dose varied within each study (12 to 50 Gy[13] and 8.5 to 20 Gy[14]), and also no control group was used.

Although our study had a larger sample size and a longer follow-up compared with other reports, there were also several shortcomings. The patients from the historical control group were not treated in the same period as the patients treated by pre-enucleation radiotherapy. The effect of the year of treatment was studied in the multivariate analysis and showed no significant association with uveal melanoma-related death ($P = .82$). Patients treated by postoperative irradiation were not excluded from the study because no effect could be demonstrated in the multivariate analysis

(P = .44). There was a significant difference in age and tumor size between the study group and control group. Both the older age as the larger tumors in the control group could have had a negative influence on survival in the control group. This can be the reason for the small difference in the Kaplan-Meier survival estimate in favor for the pre-enucleation group. After adjusting for these prognostic covariates in the multivariate analysis, this difference was minimized.

In conclusion, no long-term beneficial effect on survival could be found after 8 Gy pre-enucleation irradiation. Life prognosis in women was more favorable than in men. Longer follow-up in this study will allow the survival estimates in this comparison to stabilize. However a long-term prospective randomized trial with a larger sample size is needed to study the hypothetical effect of pre-enucleation radiotherapy.

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CHAPTER 3

METASTATIC UVEAL MELANOMA:

A morphological and immunohistochemical analysis

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ABSTRACT

Purpose: Uveal melanoma metastasizes often late and preferentially to the liver in contrast to cutaneous melanoma. The objective of the present study was to evaluate the histopathological and immunohistochemical changes in primary uveal melanomas and their corresponding metastases.

Methods: Of 29 primary uveal melanomas and their corresponding metastases, the morphology and the immunohistochemical reactivity for melanoma associated antibodies, HMB-45, S100 protein and NKI-C3, were assessed.

Results: A significant difference in cell type of the primary and the metastatic uveal melanoma was found (P=.0001). The metastases derived from the 29 patients revealed 82.5% epithelioid or highly undifferentiated cells. Positive staining of the primary uveal melanomas and the metastases was found to be 93 % and 91 % for HMB-45, 80 % and 66 % for S100, and 56 % and 71 % for NKI-C3, respectively.

Conclusions: Metastases of uveal melanomas consist of a higher grade of malignant cell types. Highly undifferentiated, non-classifiable cells can be observed in 40 % of the metastatic lesions. HMB-45 proved to be the most sensitive immunohistochemical marker in metastatic uveal melanoma and should be used as part of a panel of monoclonal antibodies in any undifferentiated metastatic tumor of unknown origin.

INTRODUCTION

Uveal melanomas metastasize relatively late: the 5, 10 and 15 year survival rates of uveal melanoma patients are 65%, 52% and 46% respectively.[1,2] These melanomas spread preferentially to the liver and once the diagnosis of hepatic metastasis clinically is made, the life-expectancy is extremely poor (2 to 7 months) and fatal in all cases.[3,4] Metastases can occur decades after treatment of the primary tumor.[1,2] Therefore, clinicians should consider metastatic uveal melanoma in the differential diagnosis of a hepatic metastasis from an unknown primary tumor.

The most important independent prognostic factors in patients with a primary uveal melanoma are cell type, largest tumor diameter (LTD), presence of closed vascular loops and age of the patient.[1,6] Presence of epithelioid cells in the primary tumor has been shown to be associated with a high mortality rate [1,2,5-8], but metastatic disease does occur in patients with a pure spindle cell melanoma and in relatively small (<3 mm thickness) tumors.[9] Though tumors of both cell types metastasize in more or lesser extent, the relationship between the cell type of the primary tumor and that of its corresponding metastases is still unclear.[10,11]

In cutaneous melanoma the sensitivity for the melanoma associated antibodies HMB-45, S100 and NKI-C3 has been reported to be 92%, 100%, and 95%, respectively.[12] In primary uveal melanomas, the immunohistochemical reactivity of these antibodies has proven to be a reliable tool in differentiating uveal melanoma from other intraocular tumors.[13-16] The S100 protein is not specific for melanocytic tumors, but it stains positively in 91% of the primary uveal melanomas.[13,14,17,18] Both the monoclonal antibodies NKI-C3 and HMB-45 bind a cytoplasmic antigen that is produced by fetal melanocytes as well as melanoma cells.[15,16,19,20] HMB-45 is sensitive in 99% of the primary uveal melanomas and is expressed most strongly at the tumor invasion front.[14,15] It may however be mentioned that HMB-45 reactivity has been demonstrated in non-melanocytic tumors but still is considered the most specific for melanocytic tumors.[21] An association between immunoreactivity of these antibodies and cell type has not been described.

It seems therefore appropriate to evaluate the morphological and immunohistochemical features of metastatic uveal melanomas in a series of cytological smears, liver biopsies and autopsy specimens.

MATERIALS AND METHODS

From the records of uveal melanoma patients, who died of uveal-melanoma-related causes, 29 cases were selected for whom the histological material of the primary tumor and its corresponding metastases was available. All available sections, paraffin blocks, and cytology slides were requested. The morphology of the primary uveal melanomas and the metastases could be studied in all 29 patients. From six autopsy cases, multiple metastatic samples from different sites were available leading totally to

40 metastases. In six out of these 40 metastases only stained cytological material was available and as a consequence only the morphology could be studied.

For immunohistochemical investigation, paraffin blocks of metastases were available in 23 out of the 29 cases. In some subjects metastatic tissue was available from more than one site, so that totally 34 metastases could be evaluated. In 16 out of these 34 metastases the paraffin blocks of the corresponding primary uveal melanoma were also available for immunochemistry.

Histopathology

From 20 serial sections of each primary tumor the predominant cell type (spindle, mixed, or epithelioid) was recorded according to the modified Callender classification.[22] The cell type of the corresponding metastases was similarly classified in a masked way. If the cells were not definable as spindle or epithelioid cell type and showed highly undifferentiated characteristics, they were called non-classifiable, or a combination of a non-classifiable and a classifiable cell type.

Immunohistochemistry

From all available formalin-fixed and paraffin-embedded blocks, sections of 6 to 7 mm were cut, mounted on aminopropyltriethoxysilane coated glass slides (APES, Sigma, St. Louis, USA), and dried overnight at 37°C to ensure tissue adherence. After deparaffinizing and rehydrating, endogenous peroxidase activity was blocked by incubation for 20 min in methanol containing 3% hydrogen peroxide. The sections were tested with three commercially available antibodies suited for formalin fixed tissue: HMB-45 (Dako, Glostrup, Denmark), S100 (Dako, Glostrup, Denmark) and NKI-C3 (Sambio, Uden, The Netherlands). They were diluted with phosphate-buffered saline (PBS); the monoclonal mouse-antihuman-melanoma antibody HMB-45 to a solution of 1:100, the polyclonal rabbit-anti-S100 antibody to 1:2500, and the monoclonal mouse-antibody NKI-C3 to 1:10. Subsequently the slides were incubated with a biotinylated secondary antibody (Biogenex; 1:50) and a streptavidin-biotin-peroxidase complex (Biogenex; 1:50). The peroxidase activity was made visible using hydrogen peroxide and 3 amino-9 ethylcarbazole as chromogene substrate. The resulting red stain allowed

easy detection of immunoreactivity in pigmented lesions. Sections were counterstained with Mayer's hematoxylin and mounted in Imsol (Klinipath, Zevenaar, The Netherlands). Negative controls were performed without the primary antibody; nerves present in the tissue served as internal positive control for the S100, and a cutaneous melanoma for the HMB-45 and the NKI-C3 antibodies. The results of the immunohistochemical stainings were graded according to the percentage of positive staining cells; negative (0), 5-25% (1), 25-50 % (2), 50-75% (3), and 75-100% (4).

Statistical analysis

In order to study the difference between the cell types of each primary uveal melanoma and their corresponding metastases, the Wilcoxon matched-pairs signed-rank test was used. The tumors were ranked from low grade malignant (spindle = 1) to high grade malignant (non-classifiable = 4), whereby in cases of multiple metastatic lesions the malignancy grade of the multiple lesions was averaged. The sensitivity for the antibodies HMB-45, S100 and NKI-C3 in both primary and metastatic lesions was assessed using the McNemar pair test. The antibodies were compared for positive staining in pairs of two. Probability level of $P < .05$ was considered significant.

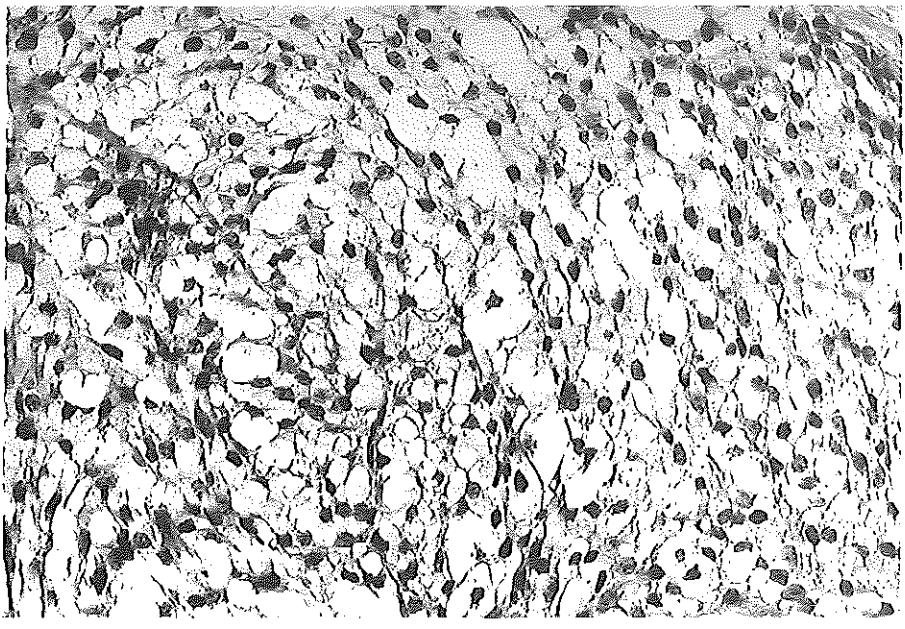

FIGURE 1. Uveal melanoma metastasis in the liver. Non-classifiable, undifferentiated melanoma cells. (original magnification x 361)

RESULTS

The distribution of the cell types of the primary and metastatic uveal melanomas of the 29 patients is shown in TABLE 1.

TABLE 1
PRIMARY AND METASTATIC UVEAL MELANOMA; CELL TYPE CLASSIFICATION AND DISTRIBUTION ACCORDING TO METASTATIC SITES

	CELL TYPE SPINDLE	MIXED	EPITHELIOID	NON-CLASSIFIABLE	TOTAL
Primary	9 (31%)	15 (51.7%)	5 (17.2%)	0 (0%)	29
Metastastic*	7 (4) (17.5%)	5 (2) (12.5%)	24 (6) (60%)	4 (10%)	40
Site					
Liver	1 (1)	3 (1)	16 (4)	3	23
Subcutis		1 (1)	4 (1)		5
Lung	2		1 (1)		3
Pleura	1 (1)			1	2
Orbit	1 (1)		1		2
Other	2 (1)	1	2		5

* main cell type (+ non-classifiable areas)

In the metastatic lesions, a significant higher percentage of high grade malignant (epithelioid and non-classifiable) cells was found than in the primary lesions (Wilcoxon matched-paired signed-rank test, p=.0001). Sixteen of the 40 metastatic lesions showed highly undifferentiated components or consisted of pure non-classifiable, undifferentiated cells (n=4)(FIGURE 1), whereas in the primary uveal melanomas no areas of these cells were observed at all. A correlation between cell type

TABLE 2

EXPRESSION OF HMB-45, S-100 AND NKI-C3 IN PRIMARY AND METASTATIC UVEAL MELANOMA

ANTIBODY	GRADE	PRIMARY UVEAL MELANOMA	METASTATIC UVEAL MELANOMA
HMB-45	0	1/15	3/32
	1	2/15	5/32
	2	4/15	5/32
	3	4/15	7/32
	4	4/15	12/32
S-100	0	3/15	12/34
	1	2/15	5/34
	2	3/15	6/34
	3	4/15	4/34
	4	3/15	7/34
NKI-C3	0	7/16	10/34
	1	0	5/34
	2	1/16	0
	3	3/16	6/34
	4	5/16	13/34

and the metastatic site could not be made because the majority of the metastatic lesions were only obtained from the liver (23/40).

The grades of immunohistochemical reactivity for HMB-45, S100 and NKI-C3 in primary and metastatic uveal melanomas are listed in TABLE 2. The percentage of positively stained cells for HMB-45 was 93 % in primary and 91 % in metastatic uveal melanomas. The percentage of positive staining for S100 and NKI-C3 was 80 % and 56 %, respectively in the primary lesions and 66 % and 71 %, respectively in the metastases. In the primary uveal melanomas, no significant differences in staining between the three antibodies could be demonstrated using the McNemar test. In the metastases, the HMB-45 was found to be significantly more sensitive than S100

(p=.0391) but not significantly more sensitive than NKI-C3 (P=.0703); no significant difference in positive staining was demonstrated between NKI-C3 and S100 (p=.6875). Of the three HMB-45 negatively stained metastatic lesions, one was found positive for both S100 and NKI-C3, whereas the two other lesions did not stain for any of the antibodies. These three HMB-45 negative metastatic lesions showed no relationship with a specific cell type; i.e. one case was of the spindle cell type, one of the mixed cell type, one of the epithelioid cell type and two with additional undifferentiated characteristics (TABLE 3). The S100 and NKI-C3 negative metastatic lesions showed no correlation with a specific cell type, although the spindle cell tumors had the highest frequency of negative staining (TABLE 3). All four metastases composed of pure non-classifiable cells showed a positive staining for HMB-45 (FIGURE 2A and 2B), three for NKI-C3 and two for S100 (TABLE 3).

TABLE 3

ANTIBODY EXPRESSION IN PRIMARY AND METASTATIC UVEAL MELANOMAS VERSUS CELL TYPE

	ANTIBODIES CELL TYPE	**HMB-45**		**S-100**		**NKI-C3**	
		+	-	+	-	+	-
Primary Uveal Melanoma	Spindle	4	0	2	2	1	3
	Mixed	8	1	8	1	6	4
	Epithelioid	2	0	2	0	2	0
	Total	14	1	12	3	9	7
Metastatic Uveal Melanoma	Spindle	5	1	2	5	2	5
	Mixed	4	1	4	1	3	2
	Epithelioid	16	1	14	4	16	2
	Non classifiable	4	0	2	2	3	1
	Total	29	3	22	12	24	10

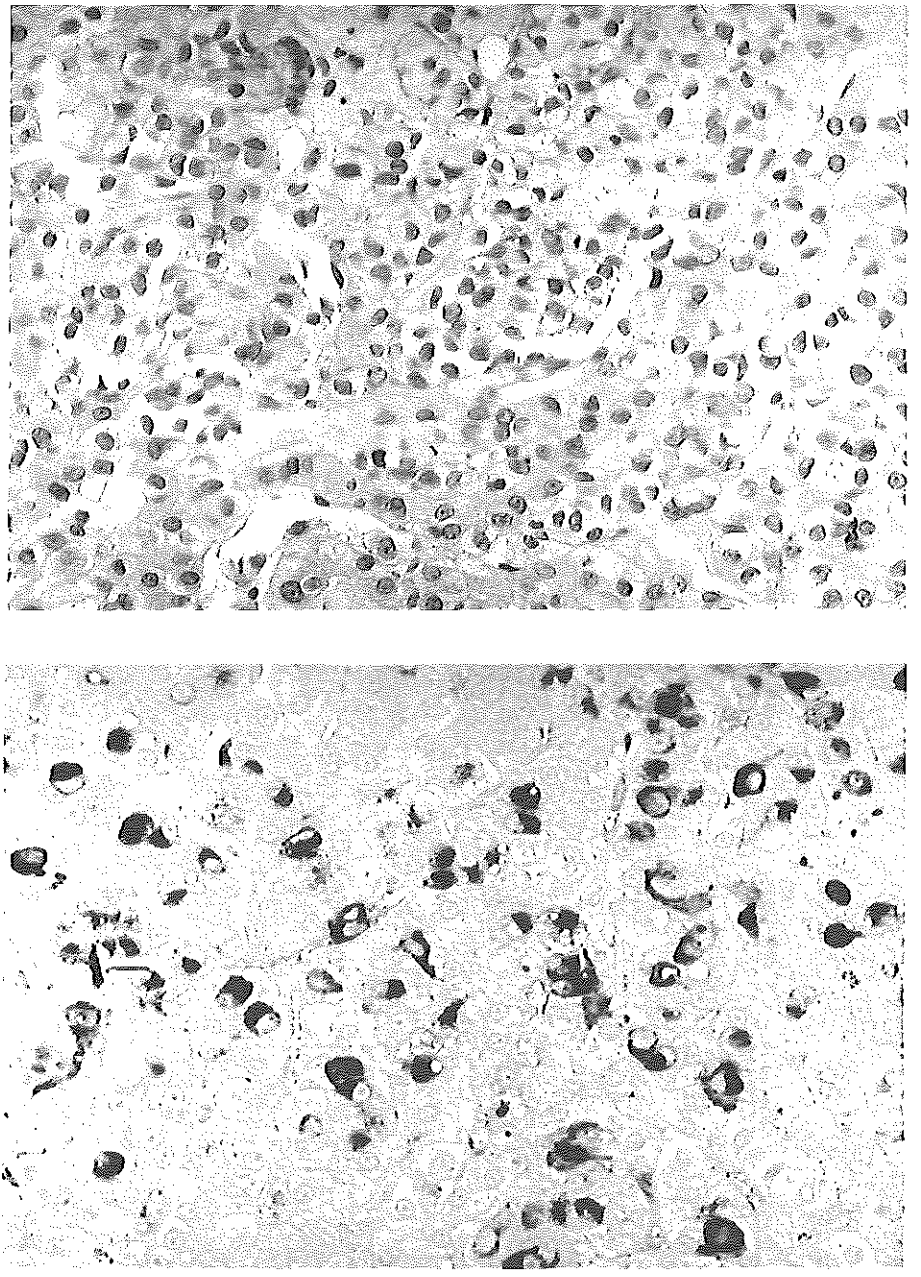

FIGURE 2A and 2B. Uveal melanoma metastasis in the liver. Undifferentiated uveal melanoma cells among normal hepatocytes (A) H/A staining (B) HMB-45 staining (original magnification x 361.)

DISCUSSION

The primary uveal melanomas in our study were selected on the basis of available specimens of metastases and showed a relatively high percentage of mixed and epithelioid cells compared to the normal distribution of cell types in primary uveal melanomas (50% spindle; 50% mixed and epithelioid).[5,23-25] Since the occurrence of epithelioid cells in primary uveal melanomas has been associated with an increased risk for developing metastatic disease in clinical studies,[1,2,5,23-25] this relatively high incidence of epithelioid cells in primary uveal melanomas in our study is probably caused by our selection criteria. Nevertheless, we found in metastatic uveal melanomas a significant higher percentage of epithelioid cells compared to the primary melanomas. This is consistent with earlier findings: Fuchs and associates[11] found in a total of 31 metastases derived from 11 uveal melanoma patients nine spindle (29 %), five mixed (16 %) and 17 epithelioid (54 %) cell types. The cell types of the 11 corresponding primary uveal melanomas were however not specified. Zakka and associates[10] found in an autopsy study on uveal melanoma no spindle, two mixed and five epithelioid cell tumors in the metastases from uveal melanomas; the primary uveal melanomas consisted of spindle cells in one case, mixed in four and epithelioid in two. The highly undifferentiated cell types observed in the present study were not described in the studies mentioned above.[10,11]

A lack of differentiation is considered to be a hallmark of malignancy. Tumor cells showing lack of differentiation are called anaplastic. Literally anaplasia means "to form backward", implying a reversion from a high level of differentiation to a lower level. However, it can be disputed whether the lack of differentiation of the non-classifiable uveal melanoma cells is the consequence of dedifferentiation or of a malignant transformation of immature melanocytes without any sign of differentiation. Therefore, these undifferentiated cells were called non-classifiable.

In our study we found four metastases that were of pure undifferentiated nature and another 12 metastases with additional non-classifiable areas next to areas with classifiable cells. These highly undifferentiated cells could not be detected in the primary tumor.

Uveal melanomas of all cell types have metastatic potency to some extent. However, in the Callender classification system (spindle, mixed and epithelioid),[22] we cannot exclude the existence of small areas of epithelioid or non-classifiable cells in a melanoma classified as predominantly spindle. Current views on tumorigenesis propose that neoplasms develop by a sequential acquisition of a number of genetic or epigenetic events, which lead to cell growth, aberrant differentiation, invasive growth, and the ability to metastasize.[26-28] This implies that tumors may arise from a single cell, i.e that they are monoclonal.[29] If the mutant subclone has a proliferative ability it may lead to overgrowth of the original or the metastatic lesion. In our study we found a high percentage of non-classifiable cells in the metastases, which may be derived from such a mutant subclone.

Furthermore, it is yet unclear whether cell-cell and cell-matrix interaction may influence the tumor cell morphology. From the present study, we could not demonstrate any correlation between cell type and metastatic site. In a study on cytokeratin expression in primary and metastatic uveal melanoma, no correlation between tumor site and cytokeratin expression could be demonstrated.[11] However, we recently demonstrated a significant difference in expression of neural cell adhesion molecules in liver metastases and other metastatic sites.[30]

In concordance with findings in cutaneous melanomas, we demonstrated that HMB-45, although not significantly different from NKI-C3, was the most sensitive marker for metastatic uveal melanoma.[12-15] In studies on cutaneous and primary uveal melanomas a high expression of S100 was found.[12-14,18] In contrast to these findings, we found a significantly lower staining for S100 in metastatic uveal melanoma; S100 will thus be less reliable in the diagnosis of difficult cases of undifferentiated metastases. The percentage of positive staining was not related to the cell type of the metastatic lesions. A non-significant higher percentage of negative staining tumors for S100 and NKI-C3 was found in the spindle cell metastatic lesions. The most aggressive cell types kept their immunoreactivity for HMB-45 and in a lesser extent for S100 and NKI-C3; all non-classifiable metastases stained positively for HMB-45.

Conclusion:

Metastatic uveal melanomas contain a significantly high percentage of epithelioid and highly undifferentiated, non-classifiable cells compared to their corresponding primary lesions. The immunohistochemical staining of HMB-45 appeared to be the most sensitive marker in characterizing metastases of uveal melanomas. Uveal melanoma may metastasize decades after diagnosis of the primary tumor. Therefore, it is important that HMB-45 should be included in the panel of monoclonal antibodies for the diagnosis of undifferentiated metastatic tumors, to identify delayed and unexpected uveal melanoma metastasis.

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CHAPTER 4

NEURAL CELL ADHESION MOLECULE DISTRIBUTION IN PRIMARY AND METASTATIC UVEAL MELANOMA

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Human Pathology 1995;11:1185-1190

ABSTRACT

Tumor cell adhesion, detachment and aggregation play an important part in tumor invasion and metastasis, and a variety of cell adhesion molecules have been found on tumor cells. Cell adhesion molecules, including those of the immunoglobulin superfamily, are associated with the development of metastatic behavior in cutaneous melanomas. The neural cell adhesion molecule (NCAM) belongs to this family. To investigate its possible role in the development metastatic behavior of uveal melanomas, we studied immunohistochemically the expression of NCAM by using an antibody that recognizes all three major isoforms of NCAM and an antibody which recognizes the HNK-1 epitope present on some isoforms of NCAM. The authors studied 32 primary uveal melanomas from 32 patients (among these, 12 were rapidly metastasizing and 16 slowly metastasizing) and 29 metastases from 19 patients. From 13 patients the primary, as well as the metastatic, tumors were available. With one exception, all HNK-1 positive primary and metastatic tumors were also positive for NCAM. NCAM was significantly more expressed in aggressive, rapidly metastasizing primary tumors ($P = .02$ and .04, respectively) and in metastases. HNK-1 was significantly ($P = .04$) more expressed in larger tumors. In liver metastases HNK-1 immunoreactivity was significantly ($P = .005$) less frequently expressed than NCAM. Therefore, NCAM isoforms which lack the HNK-1 epitope might play a role in the organ specific metastatic behavior of uveal melanomas.

INTRODUCTION

There is increasing evidence that changes in adhesiveness and motility are of considerable importance in tumor progression and may be the prime features determining aggressiveness and metastatic potential.[1] Adhesive properties of malignant cells must change repeatedly 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.[2] Adhesion molecules which comprise of several complex families, mediate intercellular interactions and interactions between cells and the extracellular matrix.[3] They are grouped into four main classes on the basis of their molecular

structure: the integrin family, the cadherin family, the immunoglobulin superfamily and the selectin superfamily.[4] The neural cell adhesion molecule (NCAM) and the intercellular adhesion molecule (ICAM) belong to the immunoglobulin (Ig) superfamily.[3,4] Although the members of the Ig superfamily are functionally divers, most are cell surface molecules involved in recognition of other surface molecules. Several neural cell adhesion molecules have been shown to mediate intercellular adhesion during the development of the nervous system, including the neural cell adhesion molecule (NCAM), myelin associated glycoprotein, L1, amalgam, contactin and fascicilin II.[5] The HNK-1 carbohydrate epitope is associated with several of these adhesion molecules, including NCAM.[6] Although the exact role the HNK-1 epitope plays is unknown, it seems to serve as a ligand in cell adhesion.[7]

For cutaneous melanoma it has been shown that the development of metastatic potential is associated with de novo expression of ICAM-1[8,9] and MUC18,[10] an antigen which shows sequence similarity to NCAM.[10,11] In contrast, others showed expression of MUC18 on a full range of benign and malignant melanocytic lesions.[12] In uveal melanoma, ICAM-1 could not be detected in one study,[13] but using a different anti-ICAM-1 monoclonal antibody (MAb) most of the uveal melanomas stained,[14,15] with a preferential reactivity of the mixed and epithelioid cell type.[15] In several human malignancies tumor progression was paralleled by changes in NCAM expression.[1,16-18] A role for NCAM in the development of malignant potential of uveal melanomas has, however, not been reported.

This study investigates whether NCAM expression is correlated with the development of metastatic potential in uveal melanoma. The authors studied primary tumors with known clinical outcome (including rapidly metastasizing and clinically non- or slowly metastasizing tumors) and all available metastases. The authors report here on NCAM, which was stained by a polyclonal antibody that recognizes all three major NCAM isoforms, and by MAb Leu-7, which recognizes the HNK-1 epitope[19] of the cell binding domain of some NCAM isoforms.[20,21]

MATERIAL AND METHODS

Patient Selection

From the files of patients with uveal melanoma related death, the authors were able to collect metastatic tissue from 20 patients (nine liver biopsies, one skin biopsy, one autopsy, nine fine needle aspirations). From 14 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. From five 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 after enucleation, irrespective of the largest tumor diameter.[22] Tumor related death within 3 years was therefore considered to be caused by rapidly metastasizing melanoma (n = 8, mean follow-up, 21,3 months) (TABLE 1). Furthermore, the authors selected nine 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 (n = 11 including two cases of tumor related death after more than 10 years) was 169.5 months.

From nine patients, the paraffin blocks of 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 years and 32 years. Four were rapidly metastasizing melanomas (TABLE 1) and five were of low metastatic potential (TABLE 2). All these patients died of tumor related death.

The total material consisted of 32 primary tumors. Among these, 12 were rapidly metastasizing (TABLE 1) and 16 slowly metastasizing (TABLE 2); the remaining four deceased due to uveal melanoma related death between three and 10 years. We investigated 29 metastases (TABLE 3) from 19 patients. Of 13 patients tissue of the corresponding primary and metastatic tumors were available.

Histology

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

Immunohistochemistry

Paraffin sections, 6 to 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. After rinsing the slides in water, antigen retrieval was performed by microwave irradiation (Bio-Rad 37°C, 750 W; 2 x 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. To detect all three major NCAM isoforms, irrespective of the presence or absence of polysialylation,[23] a polyclonal antibody was used[24] in a 30 minutes incubation in a dilution of 1 : 100 at RT. This antiboby was a generous gift from Prof. E. Bock (Research Center for Medical Biotechnology, University of Copenhagen). Visualization of antibody binding was performed as described below. HNK-1, a sulfated glucuronic carbohydrate epitope which is present on the 145 and 180 kD isoforms of NCAM and in related proteins[21,25] was detected using the anti-Leu-7 monoclonal antibody (Becton Dickinson, Sunnyvale, CA). This antibody was originally generated against a human T-cell line and is present on a subpopulation of natural killer cells,[19] but is also associated with several adhesion molecules.[20,21] This antibody was used in a dilution of 1 : 10. The slides were incubated for 30 minutes at RT with biotinylated goat-anti-mouse-rabbit-rat-guinea-pig Ig (Biogenex, San Ramon, CA) 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 hydrogen peroxide in N-N-dimethylformamide with 3-amino-9 ethylcarbazole as chromogenic 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 and tumor infiltrating lymphocytes present in the tissue section served as an internal positive control.

NCAM and HNK-1 immunoreactivity were scored semi-quantitatively as a percentage of positive cells: score 0: 0%; score 1: 0% to 5%; score 2: 5% to 50%; score 3: 50% to 100%. The immunohistochemical staining was scored without knowledge of the clinical data and was repeated after 2 months in order to assess reproducibility. Major (more than two classes) discrepancies did not occur.

Statistical analysis

Spearman's rank correlation was used to test the relation between the variables cell type, LTD and rapidly/slowly metastasizing tumors and NCAM and HNK-1 staining, respectively. Fisher's exact test was used to test correlations of NCAM and HNK-1 immunoreactivity in the metastatic sites.

RESULTS

Primary tumors

Thirteen tumors were of the spindle cell type, 15 of the mixed cell type and four of the epithelioid cell type. Four tumors were small (≤10 mm), 22 were intermediate size (10 to 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. NCAM was significantly more expressed in mixed-cell type and epithelioid-cell type melanomas (P = .02) and in rapidly metastasizing tumors (P = .04). Rapidly and slowly metastasizing tumors are summarized in TABLE 1 and 2. In the HNK-1-positive tumors, strong cytoplasmic staining was noted (FIGURE 3). HNK-1 was significantly more expressed in larger tumors (P = .04).

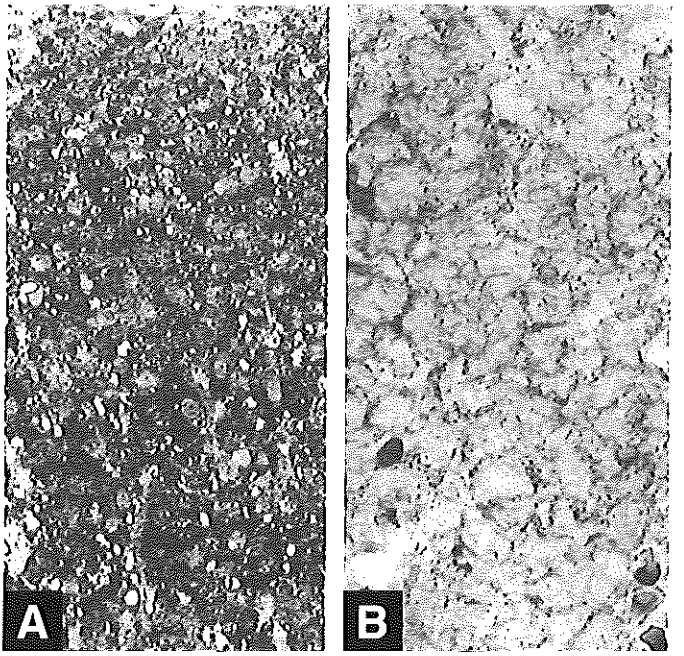

FIGURE 1A and 1B. Field of uveal melanoma of the epithelioid cell type immunostained for NCAM. (A) Cytoplasmic staining (original magnification x 361) (B) membrane-bound staining (original magnification x 880). (Hematoxylin counterstain with streptavidin-biotin-peroxidase complex immunoperoxidase with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

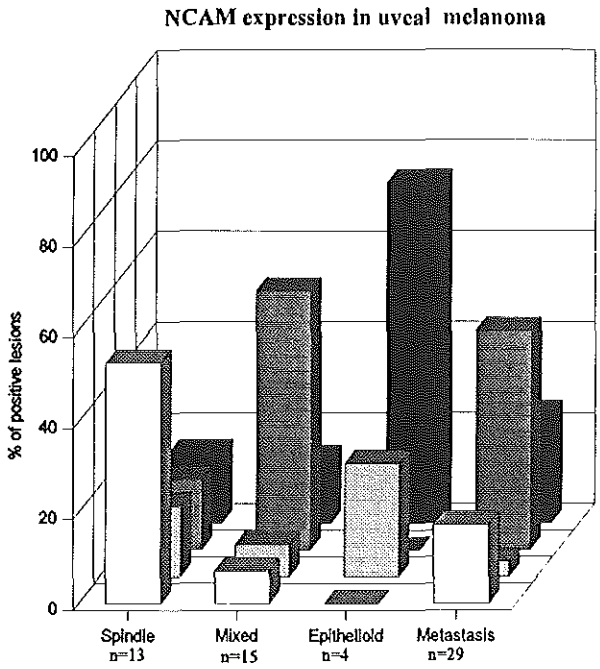

FIGURE 2. Results for NCAM immunostaining in primary and metastatic uveal melanoma. Percentage of positive cells: ■ 50% - 100%; ■ 5% - 50%; ▫ <5%; □ 0%.

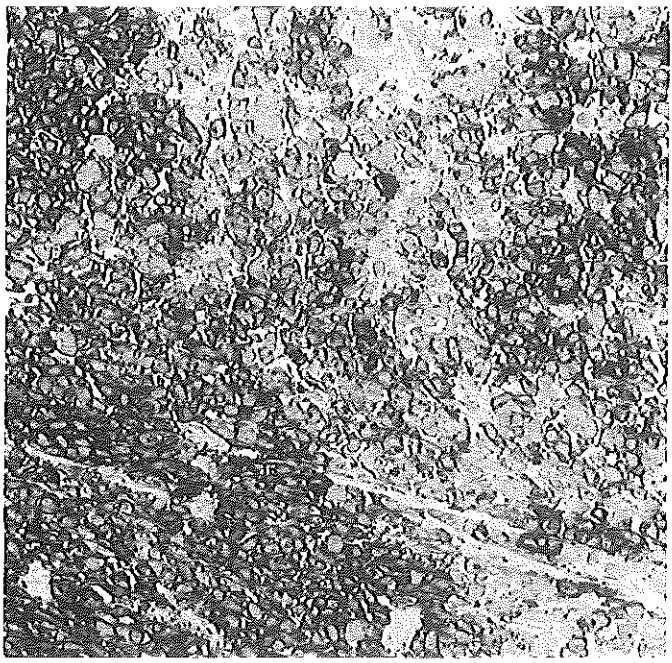

FIGURE 3. Field of metastatic uveal melanoma immunostained for HNK-1. (Hematoxylin counterstain with streptavidin-biotin-peroxidase complex immunoperoxidase with 3-amino-9 ethylcarbazole dimethylformamide substrate; original magnification x 361).

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). HNK-1 was significantly (P = .005) less expressed in liver metastases compared to other metastatic site. In five autopsies, different metastases from the same subject had a varying score (from negative to score 3) both for NCAM and HNK-1.

TABLE 1
IMMUNOHISTOCHEMICAL DATA OF MELANOMAS METASTASIZING WITHIN 3 YEARS

PAT. NO.†	CELL TYPE	LTD	PRIMARY UVEAL MELANOMA		CORRESPONDING METASTASIS	
			NCAM	HNK-1	NCAM	HNK-1
1	S	2	1	0	NA	NA
2	E	2	2	0	NA	NA
3	M	2	2	0	0	0
4	M	2	2	2	NA	NA
5	E	2	2	0	NA	A
6	M	2	NA	0	NA	NA
7	E	3	2	2	NA	NA
8	M	2	0	3	3	NA
9	M	2	3	0	NA	NA
10	M	2	2	0	NA	2
11	M	3	2	2	0	0
12	M	2	2	NA	0	NA

NOTE. Score 0 = negative; score 1 = 0% to 5% tumor cells positive; score 2 = 5% to 50% tumor cells positive; score 3 = 50% to 100% tumor cells positive.
Abbreviations: E, epithelioid cell type; M, mixed cell type; S, spindle cell type; LTD, largest tumor diameter: 1: <10 mm, 2: 10-15 mm, 3: >15 mm; NA, paraffin block not available; NCAM, neural cell adhesion molecule.
†: All patients died of tumor-related death.

TABLE 2

IMMUNOHISTOCHEMICAL DATA OF MELANOMAS
WITH A FOLLOW-UP OF MORE THAN 10 YEARS

PAT. NO.*	CELL TYPE	LTD	PRIMARY UVEA MELANOMA		CORRESPONDING METASTASIS	
			NCAM	HNK-1	NCAM	HNK-1
1	M	1	2	0	*	*
2	S	2	0	0	*	*
3	S	2	0	0	*	*
4	S	2	0	0	*	*
5	S	2	0	0	*	*
6	S	1	0	0	*	*
7	S	3	2	0	*	*
8	M	2	0	0	*	*
9	S	2	1	0	*	*
10†	S	2	2	3	NA	NA
11†	M	1	1	0	3	0
12†	S	3	3	3	2	2
13‡	S	2	0	0	2,2	3,2
14†	S	1	3	0	2	0
15‡	S	2	0	0	0,2,3,3	0,0,0,0
16†	M	3	2	0	3	0

NOTE. Score 0= negative; score 1 = 0% to 5% tumor cells positive; score 2 = 5% to 50% tumor cells positive; score 3 = 50% to 100% tumor cells positive.
Abbreviations: E, epithelioid cell type; M, mixed cell type; S, spindle cell type; LTD, largest tumor diameter: 1: <10 mm, 2: 10-15 mm, 3: >15 mm; NA, paraffin block not available; NCAM, neural cell adhesion molecule.
*: patients alive and free of metastatic disease after > 10 years;
†: tumor-related death after > 10 years.
‡: multiple metastatic sites obtained at autopsy.

Corresponding primary and metastatic tumors

Of 13 tumor pairs (partly reflected in TABLE 1 and 2), nine primary tumors and their metastases showed positive (score 2+3) staining for NCAM; in four major (negative vs positive) discrepancies between the primary tumor and the metastases were noted. For HNK-1 major discrepancies were noted in three tumor pairs. Relationship between HNK-1 and NCAM immunostaining.

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

TABLE 3

STAINING PATTERN IN THE METASTASES

	NCAM					HNK-1				
	NEG.	POS. SCORE			R	NEG.	POS. SCORE			R
		1	2	3			1	2	3	
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	22/29	20	1	6	2	9/29

* The Beta-coefficient is the natural logarithm of the hazard ratio.

† A hazard ratio, for instance 0.37 for sex, means that at each time during follow-up women have a chance of dying within one month equal to 0.37 times the chance of dying within one month for men.

DISCUSSION

Three major isoforms of NCAM have been identified, which are generated by alternative splicing of a single gene. Two of these isoforms are transmembrane proteins, differing only in their cytoplasmic domain. NCAM-140 kD has a short cytoplasmic tail, whereas NCAM-180 kD has a large cytoplasmic domain thought to interact with the cytoskeleton. NCAM-180 expression is restricted to neural tissues; the 120 kD and 140 kD isoforms also occur on other cell types.[26] One role of the different isoforms may be to target them to different cellular destinations: It has been found that glycosyl phosphatidyl inositol (GPI) membrane anchoring (small cytoplasmic domain NCAM-120) acts as an apical targeting signal in epithelia.[27] The antibody against NCAM used in our study recognizes the three major forms of NCAM, whereas MAb Leu-7 recognizes the HNK-1 epitope present in the 145 and 180 kD isoform of NCAM. Furthermore, NCAM exhibits special carbohydrate characteristics: glycosylation of NCAM seems to be regulated during development and to influence the adhesive function of the molecule.[28] 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.[29] Polysialylation of the NCAM molecule decreases its adhesion properties and may therefore play a role in connection with tumor invasion and metastasis. Immunohistochemical investigation does not provide information about NCAM polysialylation.

The percentage of NCAM positive primary tumors in our study was higher than has been reported for cutaneous melanomas.[12,17,30] This might be explained in terms of methodological differences: The authors 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.[17,30,31] NCAM was significantly more expressed in tumors with presence of epithelioid cells (mixed and epithelioid cell type). The presence of epithelioid cells is one of the factors associated with progression of uveal melanomas (development of metastatic potential).[32] That NCAM is expressed in epithelioid cells

seems surprising because epithelioid cells are morphologically noncohesive. 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 because of an increased adhesion to other cells and to extracellular 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 endothelium or extracellular matrix at a metastatic site.[16]

Immunoreactivity for HNK-1 was found in approximately one third of the NCAM positive lesions, which can be explained by the HNK-1 epitope being present on only two isoforms of NCAM. Interestingly, HNK-1 was significantly less expressed in liver metastases, compared to other metastatic sites. This discrepancy was not found for NCAM. Uveal melanomas metastasize relatively late[33,34] and in contrast to cutaneous melanomas primarily haematogenously, preferentially to the liver. Once hepatic metastases are clinically present, the median survival is extremely poor: only 2 to 9 months.[35] These findings suggest that NCAM isoforms, lacking the HNK-1 epitope might play a role in the organ-specific pattern of uveal melanoma metastasis. Isoforms with alternative modes of membrane association are targeted to different surfaces of polarized epithelial cells; the 120 kD small cytoplasmic domain is expressed on apical surface and is attached to the cell membrane via a GPI-linkage, whereas the 140 kD small surface domain form and the 180 kD large domain form are expressed on the basolateral surface.[27]

Furthermore, the authors found a significant increase of NCAM and HNK-1 positive lesions among rapidly metastasizing tumors and large tumors, and in metastases. This is in keeping with the increasing evidence that changes in adhesiveness and motility are of considerable importance to tumor progression.[1] However, a de novo expression in tumor progression could not be shown as has been found for related cellular adhesion molecules ICAM-1 and MUC-18 in progression of cutaneous melanomas.[8-10] The pattern of NCAM and HNK-1 expression in the primary tumors differed significantly from that in the paired metastases, suggesting that NCAM and HNK-1 expression are probably modulated by the tumor cell microenvironment.

In summary our results show that expression of NCAM is associated with the

development of malignant potential of uveal melanoma. Furthermore we found that NCAM isoforms, lacking the HNK-1 epitope may be associated with the organ specific metastatic behavior of uveal melanomas. The prognostic value of NCAM and HNK-1 expression in uveal melanoma remains to be established.

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CHAPTER 5

THE EXPRESSION OF NM23 GENE IN UVEAL MELANOMA

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ABSTRACT

Purpose: Low NM23-protein expression in human tumors has been associated with metastatic potential, short disease-free interval and decreased survival. We investigated the prognostic significance of NM23-protein expression in uveal melanoma.

Materials and methods: A retrospective immunohistochemical analysis was performed on 42 formalin-fixed paraffin-embedded primary uveal melanomas using a NM23/NDP kinase A antibody. The percentage of NM23-negative-staining areas was compared with conventional prognostic factors and survival. An immunocytochemical and Western blot analysis of five primary and two metastatic uveal melanoma cell lines was made in order to study the specificity of the antibody.

Results: The NM23-protein expression was not correlated with the conventional prognostic factors for uveal melanoma. The association between NM23 expression and survival was of borderline significance. The NM23-protein was expressed in the cytoplasm and to a lesser extent in the nucleus or the cell membranes. Both the NM23-H1 and H2 isoforms were found in the primary as well as in the metastatic uveal melanoma cell lines.

Conclusions: This study provides an indication that NM23 might be involved in uveal melanoma, but that the use of the anti-NM23/NDP kinase A is of limited prognostic value.

INTRODUCTION

Malignant melanomas of the choroid and ciliary body are the most common primary intraocular tumors in adults with an annual incidence of 6 per million.[1] Fifty percent of the uveal melanoma patients die within 15 years due to metastatic disease,[2] which is most frequently located in the liver.[3] Identification of genes and proteins that are involved in the process of metastasis may be useful as prognostic markers. The protein product of the *NM23* gene has been proposed to be a putative metastasis suppressor

protein. The *NM23* gene has been isolated by differential hybridization of related low and high metastatic murine melanoma sublines of the cell line K-1735.[4] Two human *NM23* isoforms have been described, known as *NM23-H1* and *NM23-H2*, which are 88 percent homologous to each other.[4,5] They have been mapped to chromosome 17q21.3-22 and were found to encode for two proteins of approximately 17 kDa.[6] These genes appear to have a sequence homology to the human nucleoside diphosphate kinase-A and -B *(NDPK-A,NDPK-B)*,[7] *NM23-H1* and *NM23-H2*, respectively. The relation between the degree of expression of the NM23 protein and metastatic potential has been most extensively studied in breast cancer. In patients suffering from breast cancer, low levels of *NM23* mRNA and NM23 protein have been found to correlate with metastases to the lymph nodes, decreased disease free intervals, and decreased relapse-free survival.[8-11] Furthermore, low levels of the NM23 protein is of prognostic significance in a variety of other tumors,[12-15] including cutaneous melanoma.[16-19]

The purpose of the present study was to investigate the prognostic value of reduced NM23 protein expression in primary uveal melanoma. To this end, the NM23 protein expression was studied in histological specimens of primary uveal melanomas and in both primary and metastatic uveal melanoma cell lines as well.

MATERIALS AND METHODS

Patients

Consecutive patients suffering from uveal melanomas of whom the eye had been enucleated between 1984 and 1992, entered this study on basis of availability of adequate histological material. The follow-up data were obtained from our database or by contacting the local ophthalmologist and/or general practitioner until December 1994. To verify date and cause of death, the files were recovered. Uveal-melanoma-related death was diagnosed by proof from biopsy material or by clinical history together with laboratory and radiodiagnostic evidence of metastatic disease. Otherwise, the patients were considered to have died from other causes. Forty-two formalin-fixed paraffin-embedded primary uveal melanomas were available. Of these, the following parameters were recorded: location (ciliary body, choroidal), cell type (spindle, mixed or epithelioid), and largest tumor diameter (LTD) of the tumor, presence of extra-scleral growth, age at the time of enucleation, gender, follow-up time and, if appropriate, cause of death.

Cell lines

The cell lines used in this study were established from five primary (EOM-3,[20,21] EOM-29[21], OCM-1,[21,22] MEL202,[23] 92-1[24]), and two metastatic uveal melanomas (OMM-1,[20,21,25] OMM-3[21]); apart from one cell line from a breast carcinoma (T47D) and one from a cutaneous melanoma (Bowes). The cell line OCM-1 was generously provided by Dr. June Kan-Mitchell (USC School of Medicine, Los Angeles, CA), while the cell line MEL202 was a generous gift from Dr. Bruce Ksander (Schepens Eye Research Institute, Harvard Medical School, Boston, USA). The T47D cell line obtained from a ductal breast carcinoma, was used as a positive control.[26] Bowes is a metastatic cutaneous cell line obtained from ATCC (CRL-9607/ U.S. patent 4, 766, 075). Six of these cell lines (EOM-3, EOM-29, OCM-1, OMM-1, OMM-3, Bowes) were cultured in a Falcon flask (3013E) with Dulbecco's-modified-Eagle's-medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (0.75 mg/ml) and streptomycin (1.25 mg/ml)(PS) at 5% CO_2 and 37°C.[19] This medium was changed twice a week and the cells

were passaged depending on their growth rate. For the other three cell lines, T47D, MEL202, and 92-1, the RPMI medium (Gibco, Life Technologies BV, Breda, The Netherlands) was used, supplemented with 10% FCS and PS. The medium of T47D was supplemented with insulin (0.2 IU/ml).

Immunohistochemistry

Paraffin sections (5 mm) were mounted on 3-aminopropyltrioxysilane coated glass slides (APES, Sigma, St. Louis, USA) and dried overnight at 37°C. After deparaffinization and rehydration, the slides were rinsed in a 0.01 M citrate buffer (pH 6.0); antigen retrieval was performed by microwave irradiation (Bio-Rad) for 2 x 5 min at 100°C. The slides were pre-incubated with normal goat serum in a dilution of 1:10 phosphate-buffered-saline (PBS) with 5% bovine serum albumin (BSA) (Fraction V, Sigma, Axel, Belgium) for 15 min. Subsequently, the slides were incubated overnight with anti-NM23/NDP kinase A (Boehringer Mannheim Gmbh, Almere, The Netherlands) in a dilution of 1:1600 PBS with 5% BSA at 4°C. After being washed in PBS/0.05% Tween 20 (PBS-T), the slides were incubated for 30 min at room temperature with biotinylated goat-anti-rabbit immunoglobulin (DAKO, Glostrup, Denmark) in a dilution of 1:50, 2% normal human serum and 2% normal goat serum in PBS with 5% BSA. After being washed in PBS-T again, the slides were incubated with streptavidin-biotin-alkaline-phosphatase complex (Biogenex) in a dilution of 1:50 for 30 min at room temperature. Finally the slides were rinsed with PBS and Tris-HCl (pH 8.0), and they were incubated in the dark in a solution of 18 mg naphtol AS-MX, 2 ml dimethylformamide, and a mix of 200 ml fuchsine, 200 ml Na_2NO_3 and 15 mg levamisol. Eventually the sections were counterstained with Mayer's hematoxylin and mounted in glycerin gelatin. As a negative control, the primary antibody was replaced by normal rabbit serum (DAKO Glostrup, Denmark; dilution 1:10). A breast cancer with an extensive in situ component served as a positive control.

Based on the hypothesis that only few cells of the primary tumor have to become metastatic competent in order to kill a patient, a scoring system was devised by Steeg et al.[27] They quantified the percentage of NM23 negative-staining ($NM23^-$) tumor cells in the area of the section with the lowest overall staining. Along the same line we screened for $NM23^-$ cells in primary uveal melanomas in a masked manner. In each section, ten fields were screened and scored for the percentage of $NM23^-$ cells.

Immunocytochemistry

The cell lines were cultured on glass coverslips until they reached half confluency. Then the coverslips were washed twice with PBS and during 10 min fixed in cold acetone (-20°C). Endogenous peroxidases were inhibited by 0.3% hydrogen peroxide in methanol for 15 min. Subsequently, the coverslips were incubated in 2% FCS/PBS for 5 min and with anti-NM23/NDP kinase A (dilution 1:20) for 135 min at room temperature. As a second step antibody, swine-anti-rabbit immunoglobulin (DAKO, Glostrup, Denmark) was used in a dilution of 1:100 and these coverslips were incubated for 60 min. Then they were incubated with 3,3'-diaminobenzidine tetrahydrochloride and ureum hydrogen peroxide (Sigma, St. Louis, USA) for 7 min, to detect the peroxidase activity. Cells were eventually counterstained with Mayer's hematoxylin for 10 seconds, rinsed with water for 10 min, dehydrated and mounted in Entelan. Between these steps the coverslips were washed with PBS-T. The NM23 protein staining was scored with a four-point scale: intense, 3; medium, 2; weak, 1; no staining, 0.

Western blot analysis

The cultured cells were washed twice with warm PBS (37°C), detached with a cell scraper and then collected in cold PBS. This cell suspension was washed with cold PBS again and sonicated, and could be stored at -20°C after addition of phenylmethylsulphonyl fluoride (PMSF) in a final concentration of 1 mM. The protein concentration of the cell suspension was determined by applying the BCA-method (Pierce, Rockford, IL, USA). Per lane 75 mg of total cellular proteins were electrophoresed in a 12% SDS-polyacrylamide gel, prepared according to the manufacturer's instructions (Biorad, Veenendaal, The Netherlands), and then transferred to a polyvinylidene difluoride membrane (Immobilon P, Millipore, Etten-Leur, The Netherlands) in a Biorad blot apparatus at 100 Volts for 2 hr. The blots were blocked by putting them for one hr at room temperature in PBS-T with 2% BSA. Subsequently, the blots were incubated overnight with NM23/NDP kinase A antibody in a dilution of 1:20 at 4°C. As a second step, peroxidase-conjugated swine-anti-rabbit antibody was used in a dilution of 1:10,000 for one hr. Following each incubation step,

blots were rinsed five times with PBS-T for 5 min. The peroxidase activity was visualized using an Enhanced Chemiluminescence (ECL) detection system (Amersham International, Little Chalfont, UK). Blots were stained with 3,3'-diaminobenzidine tetrahydrochloride and ureum hydrogen peroxide (Sigma, St. Louis, USA) for 5 min. The staining intensity was scored as in the coverslips.

Statistical analysis

The correlation between the different parameters was studied using the Spearman rank correlation test. The influence of percentage of $NM23^-$ cells and the other prognostic factors on survival was studied by performing univariate and multivariate Cox proportional hazard analysis.[28] Survival was analyzed by using the uveal melanoma-related death. Death by other causes was considered as a censored observation and thus not used in the survival analysis as death but as last date of follow-up.

RESULTS

Patient data, histopathological classification and immunohistochemistry

Fifteen out of the 42 patients died of metastatic disease before December 1994 with a total median follow-up time of 37.5 (6 - 88) months. Twenty-six patients were male and 16 female; their mean age at diagnosis was 60.8 years. The mean LTD of the 42 primary tumors was 12.6 mm; among these, there were 12 spindle cell tumors and 30 epithelioid or mixed cell tumors; we found 33 choroidal melanomas and 9 ciliary body tumors; 13 of these tumors showed extrascleral growth.

The immunoperoxidase staining revealed that the NM23-protein was localized in the cytoplasm and to a lesser extent in the nucleus (FIGURE 1). The average percentage of $NM23^-$ cells in all primary uveal melanomas was 78%.

Immunocytochemistry and Western blot analysis

The immunocytochemical NM23 staining intensity of the uveal melanoma cell lines is shown in TABLE 1. The staining pattern was again mainly cytoplasmic and to a lesser extent staining was observed in the nucleus or membranes (FIGURE 2).

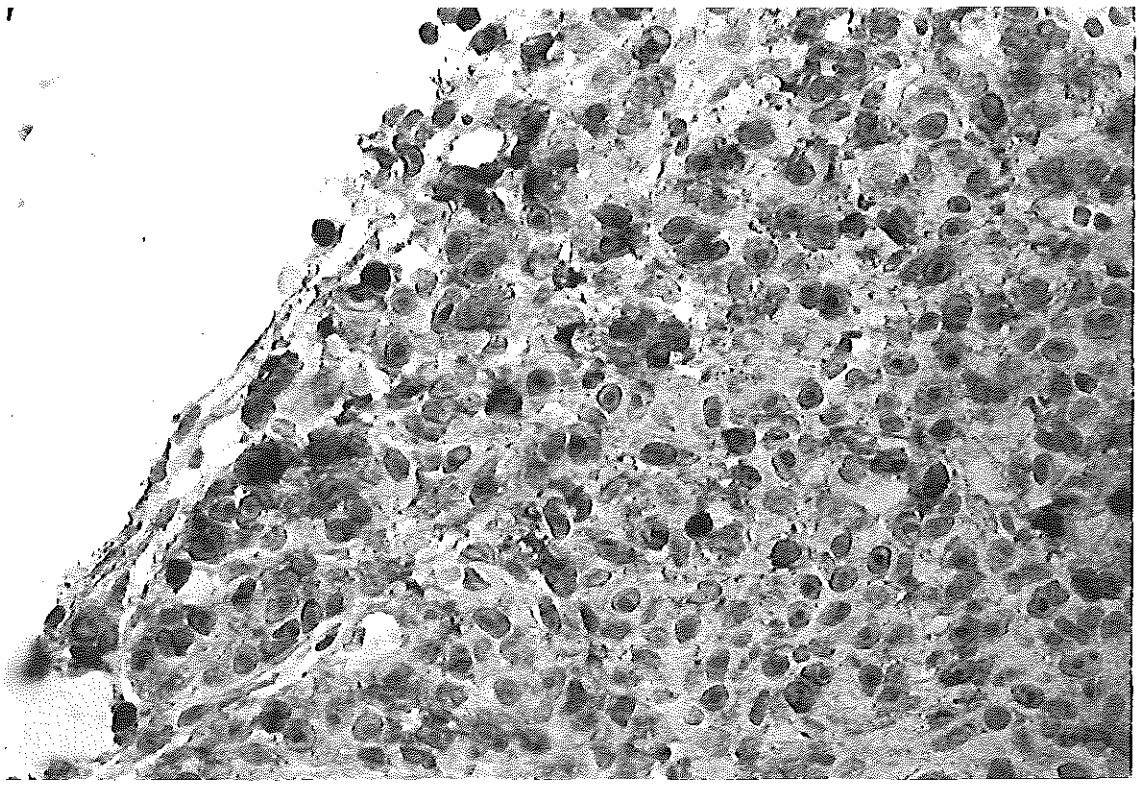

FIGURE 1. Primary uveal melanoma stained with anti-nm23/NDP kinase A. Positive nm23-staining cells in a primary uveal melanoma.

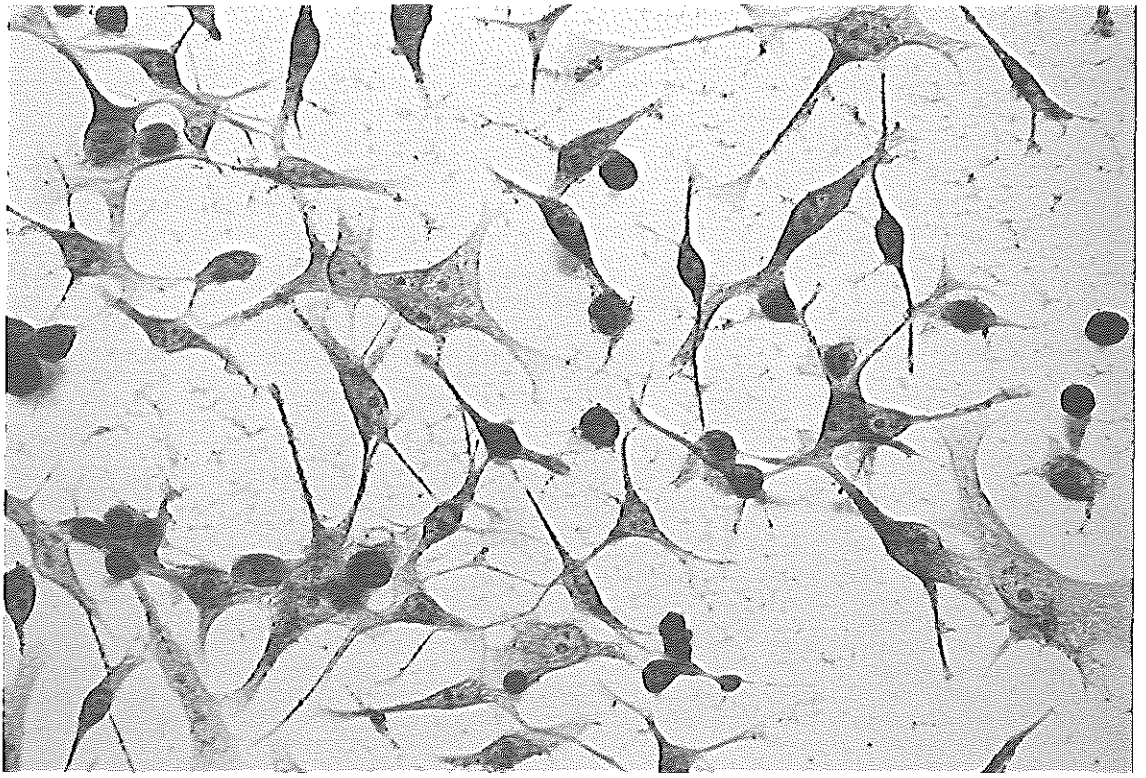

FIGURE 2. Cytoplasmic and nuclear staining of nm23 in uveal melanoma cells (OMM-1) on coverslips.

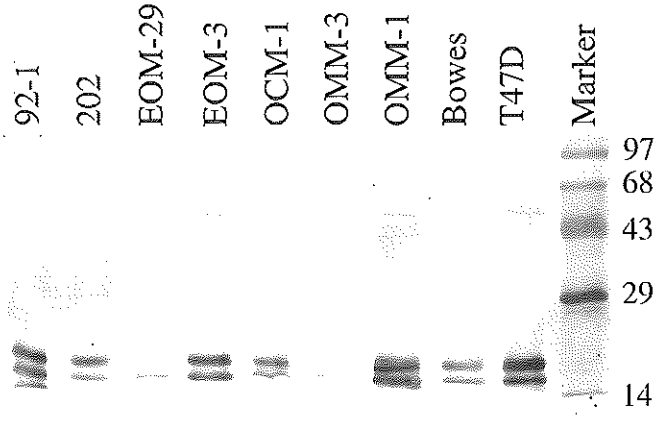

FIGURE 3. Western blot analysis of the cell lines; lane 1, 92-1; lane 2, Mel202; lane 3, EOM29; lane 4, EOM-3; lane 5, OCM-1; lane 6, OMM-3; lane 7, OMM-1; lane 8, Bowes; lane 9, T47D.

TABLE 1

NM23 EXPRESSION IN CELL LINES

CELL LINE	ORIGIN OF TUMOR	EXPRESSION OF NM23 WITH ANTI-NM23/NDP KINASE A	
		CYTOCHEMISTRY	WESTERN BLOT
92-1	UM	3	3
Mel202	UM	3	2
EOM29	UM	2	1
EOM-3	UM	3	3
OCM-1	UM	1	2
OMM-1	MUM	3	3
OMM-3	MUM	3	1
T47D	Breast carcinoma	3	3
Bowes	nodal metastasis cutanous melanoma	1	1

UM: uveal melanoma (primary); MUM: metastatic uveal melanoma
Scoring: Cells on coverslips were scored according to cytoplasmic staining intensity. A four-point scale was used: intense staining, score 3; medium staining, score 2; weak staining, score 1; no staining, score 0. The same scoring scale was used for the Western blot analysis.
nd: not done

All cell lines clearly expressed the NM23 protein except for two cell lines (OCM-1, Bowes) that showed only a weak staining.

The Western blot analysis of the extracts of the cell lines of seven uveal melanomas, one breast carcinoma and one cutaneous melanoma, as determined by the NM23/NDP kinase A antibody is shown in FIGURE 3. Two bands (M_r 17 kD and M_r 18.5 kD) could be detected in all cell lines; in three (92-1, OCM-1, and OMM-1) a third band of M_r 15 kD was seen (lane 1, 5 and 7). Their staining intensity is shown in Table I. Extracts of the cell lines 92-1, EOM-3, OMM-1, T47D showed two intensively NM23 staining bands (lane 1, 4, 7 and 9); those of MEL202, OCM-1, and Bowes a less intensive (lane 2, 5 and 8), whereas in those of EOM-29 and OMM-3 only a weak staining could be detected (lane 3 and 6).

TABLE 2

SPEARMAN RANK CORRELATION COEFFICIENT (r_s)
OF THE EXAMINED PARAMETERS.

	PERCENTAGE OF NM23- CELLS	
	r_s	P-value
Cell type*	.15	.32
Extrascleral growth	-.09	.57
LTD†	.05	.74
Location‡	-.04	.78
Gender	.05	.77
Age	-.03	.86

* Cell type (spindle cell/ mixed and epithelioid)

† Largest Tumor Diameter

‡ Tumor location (choroid/ciliary body)

TABLE 3

COX PROPORTIONAL HAZARD ANALYSIS; UNI- AND MULTIVARIATE ANALYSES

VARIABLE CORRECTED FOR	BETA COEFFICIENT†	STANDARD ERROR	P VALUE	HAZARD RATIO‡
nm23*	.033	.017	.057	1.034
Age	.037	.019	.048	1.037
Gender	.037	.018	.040	1.038
Extrascleral growth	.033	.017	.059	1.033
Tumor location	.034	.018	.060	1.035
Largest tumor diameter	.035	.019	.068	1.035
Cell type	.032	.018	.074	1.032

* Univariate analysis

† The Beta-coefficient is the natural logarithm of the hazard ratio.

‡ A hazard ratio, for instance 1.034 for nm23, means that at each time during follow-up patients with an uveal melanoma with nm23- staining areas have a chance of dying within one month equal to 1.034 times the chance of dying within one month for patients with nm23+ staining areas.

Statistical analysis

The results of the Spearman rank correlation test are shown in TABLE 2. No correlation between the percentage of $NM23^-$ cells and the other clinical and histopathological parameters could be demonstrated.

In the Cox proportional hazard univariate analysis (TABLE 3), no significant association between survival and percentage of $NM23^-$ cells could be demonstrated (P=.057). Because of the relatively small sample size and therein the small number of uveal melanoma-related deaths (n=15), only two parameters could be included simultaneously in one multivariate analysis. The results of the multivariate analysis of the clinical and histopathological parameters with the $NM23^-$ expression showed a significant prognostic value for the $NM23^-$ expression after adding age (P=.047) and gender (p=.040) to the multivariate analysis. However no significance was found by adding extrascleral growth, tumor location, LTD and cell type.

DISCUSSION

Although the prognostic value of NM23/NDPK has been demonstrated in various human tumor types using immunohistochemical or mRNA expression,[8-19] other research groups found conflicting results showing no correlation between the NM23/NDPK expression and survival.[29,30] In two other tumor types even increased levels of NM23 expression have been found in tumors with a high grade of malignancy.[31,32] In cutaneous melanoma, reduced *NM23* expression on mRNA level has been reported in aggressive tumors.[17,18] The *NM23* mRNA expression was variable in metastatic lesions of cutaneous melanoma,[17,19] while low mRNA levels in the primary lesion showed a significant correlation to a reduced disease-free interval.[17,20] These results found in cutaneous melanoma suggested a promising role of the NM23/NDPK expression as a prognostic factor in uveal melanomas. The present study of primary uveal melanomas showed no correlation between the NM23-protein expression, using the NM23/NDP kinase A antibody, and the other prognostic factors. Even the most independent prognostic factors in uveal melanoma, such as LTD, cell type and age of the patient, showed no correlation with NM23/NDP kinase

A expression. A borderline significant association could be demonstrated between the percentage of negative staining tumor cells for the NM23/NDP kinase A antibody and survival in uveal melanoma patients in the univariate and multivariate analysis. The relatively small sample size of the study limited the multivariate analysis to include only two parameters. In a study with a large sample size, the inverse relation between NM23-expression and survival could possibly more significant.

In the present study we have used a polyclonal antibody to NM23/NDP kinase A and this antibody showed immunoreactivity mainly in the cytoplasm and in a lesser extent in the nucleus, which is in agreement with other reports.[27] This antibody was chosen because it should recognize only the NM23-H1 isoform. The detection of the NM23-H1 isoform was found to be of more significance to demonstrate the inverse association between the NM23 and survival.[12,27] However the NM23/NDP kinase A antibody we have used in our experiments showed cross-reactivity with the NM23-H2 isoform. The Western-blot analysis demonstrated both NM23 isoforms, NM23-H1 and H2. The M_r 18.5 kD and M_r 17 kD bands that showed up are identical to the described positions of the NM23-H1 and NM23-H2 proteins.[27,33] Even a third band of M_r 15 kD was found in three cell lines, but this could possibly be the result of the high amount of protein extracts (>50 µg) used in the experiments.[27] It will probably important to use more selective antisera, which recognize specifically the NM23 isoforms. Whether this could also be the reason that we could not find a difference in NM23 expression between the primary and metastatic cell lines by the immunocytochemical and Western blot analysis can be questioned. A more selective mouse monoclonal antibody, that was used by Ma et al.(manuscript in preparation)[34], showed a comparable NM23 protein expression in the cell lines EOM-3, MEL202 and OCM-1. A discordance however was found in cell line 92-1. Although we found a high NM23 protein expression in the cell line 92-1, Ma et al.[34] found only weak immunohistochemical expression of NM23/NDPK-A antigen. This discordance in NM23-protein expression in cell line 92-1 can possibly explained by the selectivity of the two different antibodies or indeed by in vitro selection of the melanoma cells, which can be the result of the culturing technique.

The exact biological role of *NM23* as a metastasis suppresser protein is still lacking.[9,27] Nucleoside diphosphate kinases (NDPK) activity of the NM23 protein has

been proposed to be related to the anti-metastatic effect. However, it has shown that the NDPK activity was not correlated with NM23 protein levels nor with the anti-metastatic effect.[33] Evidence of its metastasis suppressor function has since been found to be on another level: firstly, on the post-transcriptional level in the role of autophosphorylation of the serine instead of histidine residue of the NM23-protein. The acid-stable phosphoserine does correlate with suppression of tumor metastatic potential.[33] Secondly, another presumed biochemical activity of the NM23 protein (NM23-H2) has been found on transcription level. In vitro studies on the transcriptional regulating function of NM23 have shown that the NM23-H2 isoform exhibits a purine binding factor (PuF) which is able to bind to the oncogene *MYC* and as a consequence is able to regulate its transcription.[35] *MYC* overexpression has been shown to correlate with proliferation and metastatic potential in general and has been demonstrated in uveal melanoma as well.[35,36] Whether the transcriptional regulating function of NM23-H2 acts separately from its NDPK activity and is correlated with the anti-metastatic function is as yet unknown.[37]

In conclusion, NM23 protein expression using the NM23/NDP kinase A antibody has limited prognostic value, but indicated a possible role of the NM 23 gene in uveal melanoma. The exact biological role of the NM23-protein is still unclear. Further studies on post-transcriptional and transcription level of the NM23-protein are necessary to investigate the relationship between expression of NM23 and metastatic potential of uveal melanoma.

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CHAPTER 6

ESTABLISHMENT AND CHARACTERIZATION OF PRIMARY AND METASTATIC UVEAL MELANOMA CELL LINES.

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ABSTRACT

We report on the establishment and characterization of two primary (EOM-3, EOM-29) and three metastatic uveal melanoma cell lines (OMM-1, OMM-2, OMM-3), and further cytogenetic characterization of a previously described primary uveal melanoma cell line (OCM-1). Only a few long-term growing primary uveal melanoma cell lines have as yet been established, while of metastatic uveal melanoma cell lines we have found no descriptions. The morphology of the *in vitro* cultured cells varied from spindle to epithelioid. The cell lines were characterized by immunocytochemistry, electron microscopy and cytogenetical analysis. The relative growth rate was determined by bromodeoxyuridine (BrdU) incorporation. The melanocytic origin of the cell lines was determined by the positive staining with antibodies identifying melanoma associated antigens. Melanosomes and pre-melanosomes were indeed observed by electron microscopy in all cell lines. The stem cell karyotype was found to be normal in three cell lines (EOM-29, OMM-2, OMM-3) and abnormal in three others (EOM-3, OCM-1, OMM-1) showing a net loss of chromosome 6. The OCM-1 and the OMM-1 cell lines even demonstrated a large amount of structural chromosomal aberrations, the former being near tetraploid and the latter triploid. The EOM-29 cell line, cultured from an ciliary body melanoma, did not show the previously described chromosome 3 and 8 abnormalities.

INTRODUCTION

Uveal melanoma is the most common primary intraocular tumour in adults.[1] These tumours spread haematogeneously and preferentially to the liver. A 50 % overall incidence of metastasis occurs within 15 years after initial treatment by enucleation or radiotherapy of the tumour-containing eye.[2] After clinical diagnosis of hepatic metastases, median survival is extremely poor: between two and seven months.[2] The biological and molecular properties of the successive steps involved in uveal melanoma progression and metastasis could be studied in detail once established uveal melanoma cell lines were available. A limited number of cell lines obtained

from primary uveal melanoma have indeed been described,[3-6] but not from metastatic uveal melanoma.

In this study, we report on the successful establishment of two primary (EOM-3, EOM-29) and three metastatic uveal melanoma cell lines (OMM-1, OMM-2, OMM-3) in our institute. These cell lines were characterized by immunocytochemistry, light and electron microscopy, and cytogenetic analyses. The relative growth rate was determined by bromodeoxyuridin (BrdU) incorporation. In addition, a previously described primary uveal melanoma cell line, OCM-1,[3] was further characterized by extended karyotypic analysis.

MATERIAL AND METHODS

Tumour material

After enucleation of the uveal melanoma containing eye, part of the ocular tumour was taken for cell culture. The eye was further histologically examined to confirm the diagnosis. When, during follow-up of the patient, metastatic disease was suspected, a biopsy was taken for diagnosis and for cell culture. All patients had given full informed consent. Between January 1992 and May 1993, cells of the primary tumours of 16 patients and of the metastatic tumours of four patients were processed for culture. The patients with primary uveal melanoma ranged in age from 22 to 87 (mean: 56.8); nine were female and seven were male; eight patients had a choroidal and eight patients had a ciliary body melanoma; three patients had a spindle-cell tumour, nine had a mixed-cell tumour and four had an epithelioid-cell tumour; tumour diameter ranged from 3 to 22 mm (mean: 11.6 mm). The patients were followed until May 1995; two patients died of metastatic disease and one is alive, 6 months after detection of a metastatic lesion in the subcutis. From all four patients with metastatic disease tumour material was obtained from sub-cutaneous lesions. From one patient the primary as well as the metastatic tumour were available.

Culturing methods

The tumour material was processed and cultured within one to three hours after dissection as described by Luyten *et al.*[7] In brief, the tumour-containing eye or the metastatic lesion was dissected under sterile conditions. A full thickness biopsy was taken and transported in Dulbecco's-modified-Eagle's medium supplemented with 10% fetal calf serum, and 1% penicillin/streptomycin (full DMEM). The tumour material was suspended with a small pair of scissors and strained through a linen cloth by continuous irrigation with full DMEM. The resulting suspension of single cells and to a lesser extent of small clumps of tumour cells, was then washed two or three times with full DMEM. This washing was repeated depending on the amount of pigment. The cells were seeded in a culture flask (Falcon plastic T-30) with 5 ml full DMEM at 5% CO_2 (37°C). After one week the medium was renewed while the non-attached cells were discarded. Subsequently, the culture medium was changed twice a week. At reaching confluence, the cells were detached with 0.05% trypsin and 0.5 mM Na_2 EDTA and subsequently subcultured. Depending on the growth rate the cultures were passaged at 1:2 to 1:10 dilutions.

BrdU incorporation

To measure the logarithmic growth rate of the cells in culture, the cells were incubated in culture flasks for six hours with 1 mM BrdU (Boehringer Mannheim, Almere, The Netherlands). After being washed and detached, the cells were centrifuged onto object glasses, fixed in 2 M HCL at 37°C for 30 minutes and then incubated in 0.1 M borate buffer pH 8.5 at room temperature for five minutes. The cells were subsequently washed twice with PBS and incubated for 30 minutes with a peroxidase-conjugated immunoglobulin of a mouse monoclonal antibody specific for BrdU (Boehringer Mannheim; dilution 1:33) in 1% BSA/PBS. After washing with PBS again, the incorporated BrdU was visualized with 3'3 diaminobenzidine and ureum hydroxide (Sigma, St. Louis, MO). The percentage of BrdU incorporated cells could then be counted in one low power field (10X) using an eye piece grid.

Immunocytochemistry

The cells were cultured on a coverslip, washed twice with PBS at 37°C and subsequently fixed in cold acetone at -20°C for 10 minutes. Endogenous peroxidase was inhibited by incubation in 0.3% hydrogen peroxide/methanol for 15 minutes. Subsequently the coverslip was incubated in 2% fetal calf serum/PBS for five minutes. The primary antibodies, S100 (Dako, Glostrup, Denmark), NKI-C3 (Dako), HMB-45 (Biogenex, San Ramou, CA) and HNK-1 (Becton Dickinson, San Jose, CA) were incubated at dilutions of 1:600, 1:20, 1:50, 1:10, respectively for 1 hour. For S100, swine anti-rabbit peroxidase-conjugated immunoglobulin was used as a second step antibody (Dako; dilution 1:100). For the other three antibodies rabbit anti-mouse peroxidase-conjugated immunoglobulin (Dako; dilution of 1:100) was used. Both secondary antibodies were incubated for one hour. The activity of the peroxidase was then visualized using tablets of 3,3' diaminobenzidine HCL and hydrogen ureaperoxide (Sigma). Finally, the cells were counterstained with haematoxylin for 10 seconds. Between each of the incubation steps the coverslip was rinsed with 0.1% Tween-20/PBS.

Antibodies

The S100 antibody recognizes an acidic intracellular Ca^{++} binding protein and is a sensitive but non-specific marker for melanoma cells.[8] Monoclonal antibody NKI-C3 and HMB-45 binds both a cytoplasmic antigen produced by fetal melanocytes and melanoma cells of adults.[9,10] The monoclonal antibody HNK-1 recognizes a family of cell adhesion molecules (CD 57), migrating neural crest cells and a series of neural crest derivatives including tumours of neural crest origin.[11]

MHC class I expression

Sub-confluent cell cultures were detached with trypsin/EDTA, after which cytocentrifuge slides could be prepared. The major histocompatibility complex (MHC) class I expression was dertermined using the standard NIH micro-cytocytoxity test with locally obtained reagents.

Electron microscopy

After detachment by use of trypsin/EDTA, the cultured cells were centrifuged and fixed in 3% glutaraldehyde at room temperature for 30 minutes. Then they were post-osmificated for 30 minutes in 1% OsO4, and subsequently embedded in Epon. Ultrathin sections were prepared and counterstained with uranyl acetate and lead citrate.

Cytogenetic analysis

In order to obtain metaphases, cultured cells in the logarithmic phase of proliferation were treated with colcemid for one to three hours. After trypsinization, the cells were subjected to a hypotonic (0.075 M KCL) solution. Finally the cells were gradually fixed with cold methanol/acetic acid (3:1 v/v). Air-dried slides were banded by the reverse method.

To identify the marker chromosomes, fluorescence in situ hybridization (FISH) was used. Slides were then hybridized with chromosome-specific libraries,[12] kindly provided by Dr. J. Gray. The results presented conform to the recommendations of the International System for Human Cytogenetic Nomenclature (ISCN).[13]

RESULTS

Primary and metastatic uveal melanoma cell lines

Out of the 16 cultures of the primary uveal melanomas, two cell lines could be established successfully. The remaining cell cultures stopped growing after being passaged two to five times. Of the cultures of the metastatic uveal melanomas, three out of four resulted in a stable growing cell line. The cell cultures from one patient, from whom both the primary and corresponding metastatic tumour were available, were not successful. The patient data and tumour data from the established cell lines are summarized in TABLE 1.

TABLE 1

PATIENT AND TUMOUR DATA OF SUCCESSFULLY ESTABLISHED UVEAL MELANOMA CELL LINES

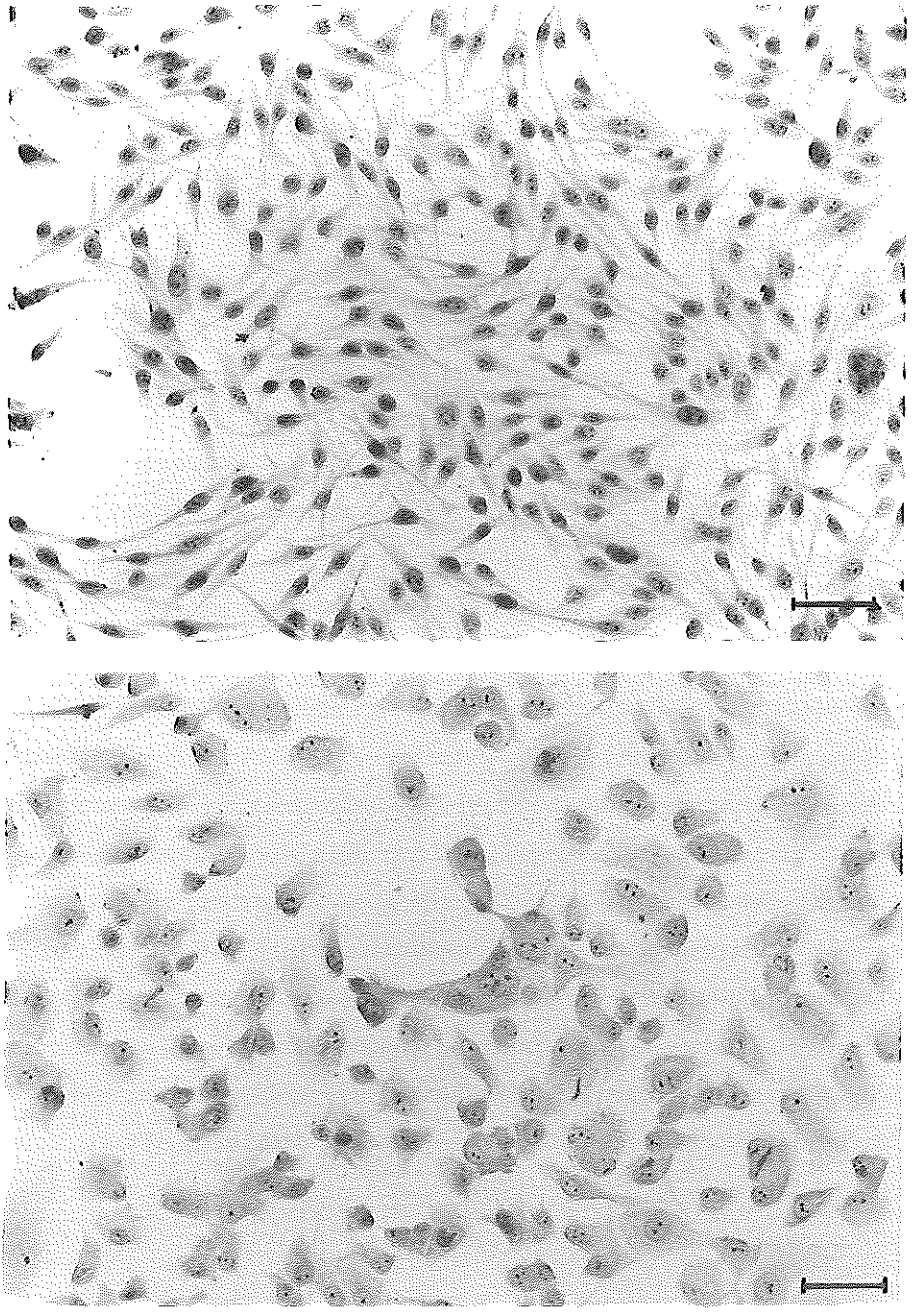
CELL LINE	PATIENT SEX / AGE	SURVIVAL(MO)	TUMOUR LOCATION	HISTOLOGY	CELL LINE MORPHOLOGY(LM)	PASSAGE
OCM-1*	F / n.a.	n.a.	Posterior	Spindle B	Mixed	>50
EOM-3*	M / 62	29	Posterior	Mixed	Epithelioid	20
EOM-29*	M / 87	14	Ciliary body	Epithelioid	Spindle	15
OMM-1†	M / 74	343‡	Subcutis	Mixed	Mixed	42
OMM-2†	M / 72	40‡	Subcutis	Epithelioid	Spindle	23
OMM-3†	M / 72	41‡	Subcutis	Mixed	Spindle	19

* Primary uveal melanoma cell line
† Metastatic uveal melanoma cell line
‡ Tumour related death
Abbreviations: F, female; M, male; n.a. = not available

The primary uveal melanoma cell line OCM-1, described by Kan-Mitchell and associates,[3] has been growing for more than seven years without morphological changes (FIGURE 1A). The EOM-3 cell line was established from a mixed cell type primary uveal melanoma. During the first six passages, the cultured cells had a spindle cell type appearance. However, a small clone of epithelioid cells started to grow and subsequently overgrew the rest of the tumour cells. The epithelioid-cell culture underwent no further morphological changes and has been growing now for two years (FIGURE 1B). EOM-29 was established from a large extra-ocular extension of a ciliary body melanoma (epithelioid cell type) and could be passaged once a week. Gradually the passage time increased with a decreasing proliferation rate. After passage ten, the cells had a spindle like appearance in culture (FIGURE 1C) and continued to grow without demonstrating changes in their morphology.

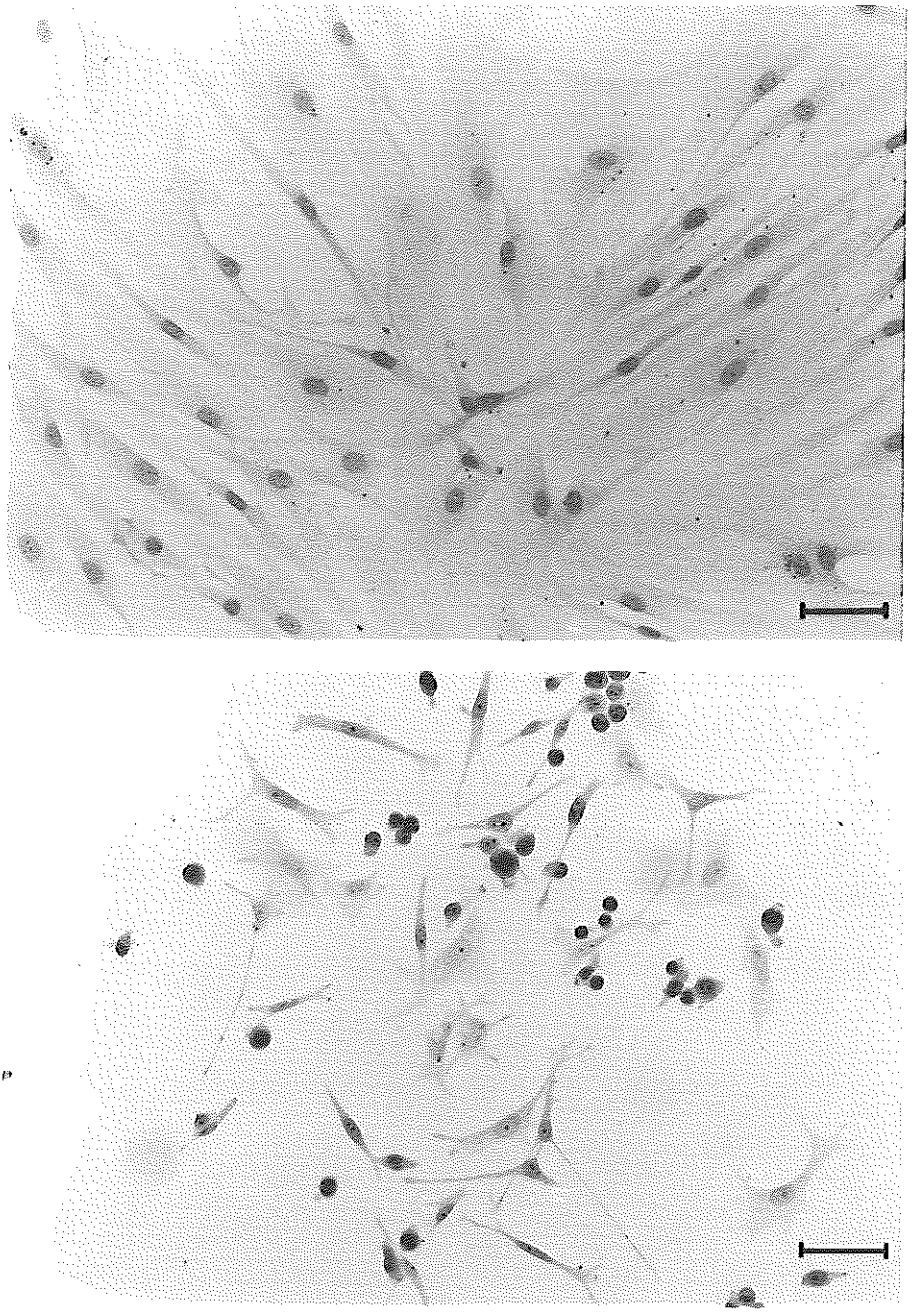
A subcutaneous metastatic lesion (mixed cell type) was excised from a uveal melanoma patient (OMM-1), who had undergone an enucleation of his primary tumour 29 years earlier. The primary culture of this metastatic lesion and the first ten passages consisted of adherent and non-adherent cells. Gradually more cells became adherent, showing a heterogeneous morphology of spindle and epithelioid cells. This morphological heterogeneous spectrum remained the same over 2.5 years (passage 42) (FIGURE 1D). The primary culture (OMM-2) of a sub-cutaneous metastatic lesion (epithelioid-cell type) had a spindle cell pattern in cell culture, and did not show morphological changes for two years. The cells have been growing slowly but continuously with a passage time of one month (FIGURE 1E). The most recently established metastatic cell line (OMM-3) was cultured from a large, partly amelanotic sub-cutaneous metastatic lesion (mixed-cell type) with a spindle cell appearance in cell culture. This cell line has now been growing for more than one year and no morphological changes have been observed (passage 19) (FIGURE 1F).

FIGURE 1 Light microscopy (scale bar, 50 mm).



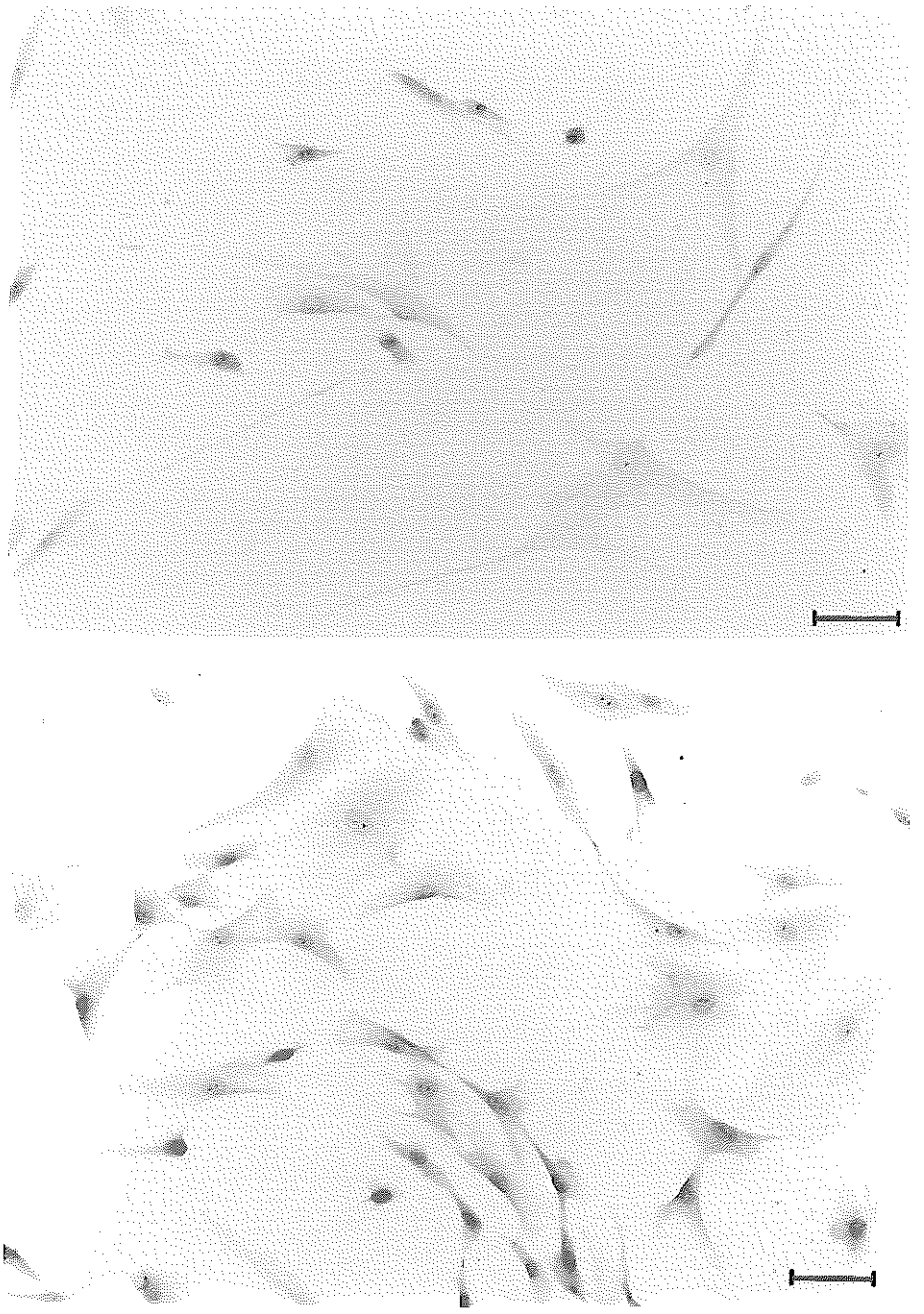
(A) The OCM-1 cell line has small and large rounded cells, with one or more nuclei, growing upon a stellate and elongated cell layer with dendritic extensions;

(B) the EOM-3 cell line shows a monolayer of plumb polyhedral anaplastic, epithelioid-cells with abundant cytoplasm, large nuclei and prominent nucleoli;



(C) The EOM-29 cell line forms spindle like patterns with large round nuclei;

(D) The OMM-1 cell line: cluster of large round cells (mono- and multi-nucleated), irregularly shaped stellate and elongated cells. Very large cells with abundant cytoplasm and cytoplasmic inclusions were observed;



(E) The OMM-2 cell line showed a monolayer characterized by predominantly elongated cells with large nuclei, abundant cytoplasm and long dendritic extensions, forming a bundle-like, spindle cell pattern;

(F) OMM-3 cell line has spindle-cell appearance with dendritic extensions; The nuclei are large with abundant cytoplasm and prominent nucleoli.

Electron microscopy, immunocytochemistry

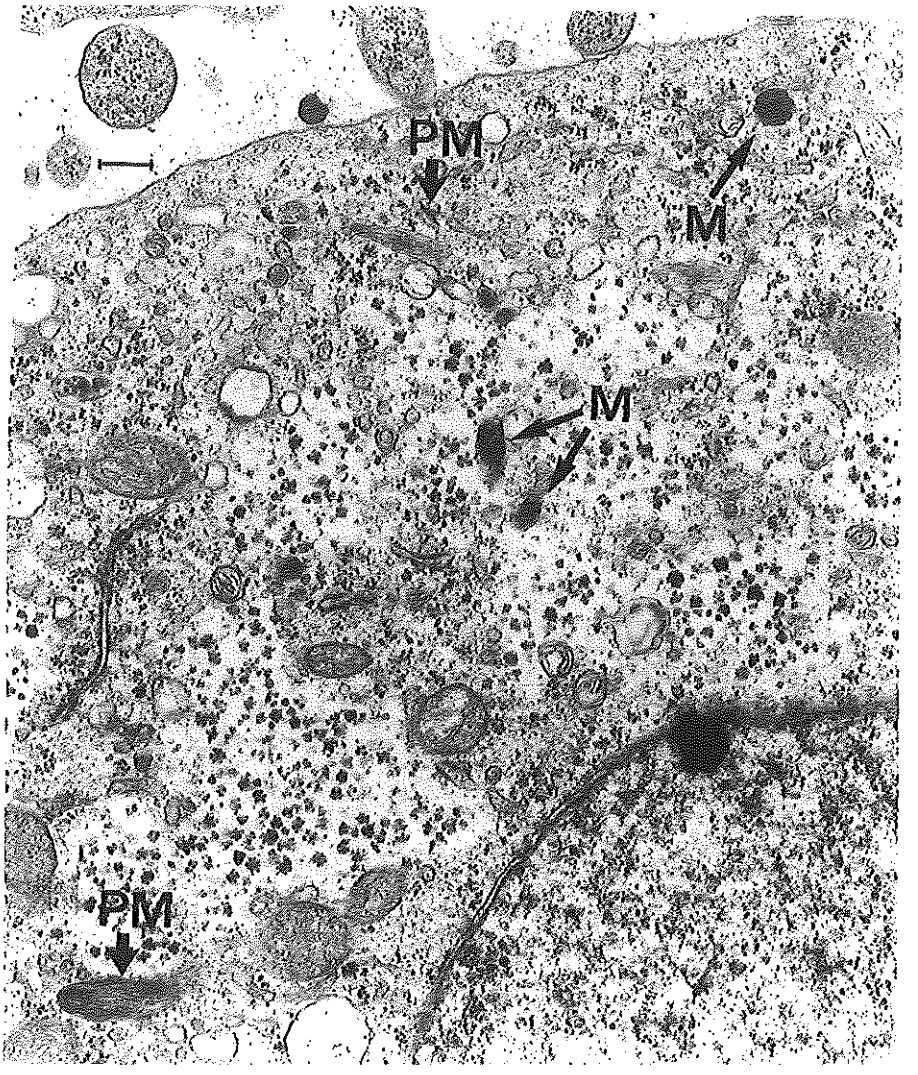
Electron microscopical morphology of the six cell lines is shown in FIGURE 2A-F. All figures are representative for the cultured cells examined and contained melanosomes and pre-melanosomes (TABLE 2). The immunocytochemical staining of the five cell lines with NKI-C3, HMB-45, S100, HNK-1 and BrdU incorporation is summarized in TABLE 2. The NKI-C3 stained all five cell lines; expression of the other three markers appeared to be facultative. The cell lines EOM-3 and OMM-1 showed a relatively high BrdU incorporation, thus showing a high frequency of cell division. The more slowly growing cell lines EOM-29, OMM-2 and OMM-3 had indeed a lower BrdU incorporation. The primary cell lines OCM-1 and EOM-29 were found to express MHC-class-I molecules, whereas in the EOM-3 cell line, no detectable MHC-class-I expression was observed. All three metastatic cell lines were found to express MHC-class-I antigens.

TABLE 2
CHARACTERISTICS OF THE ESTABLISHED CELL LINES

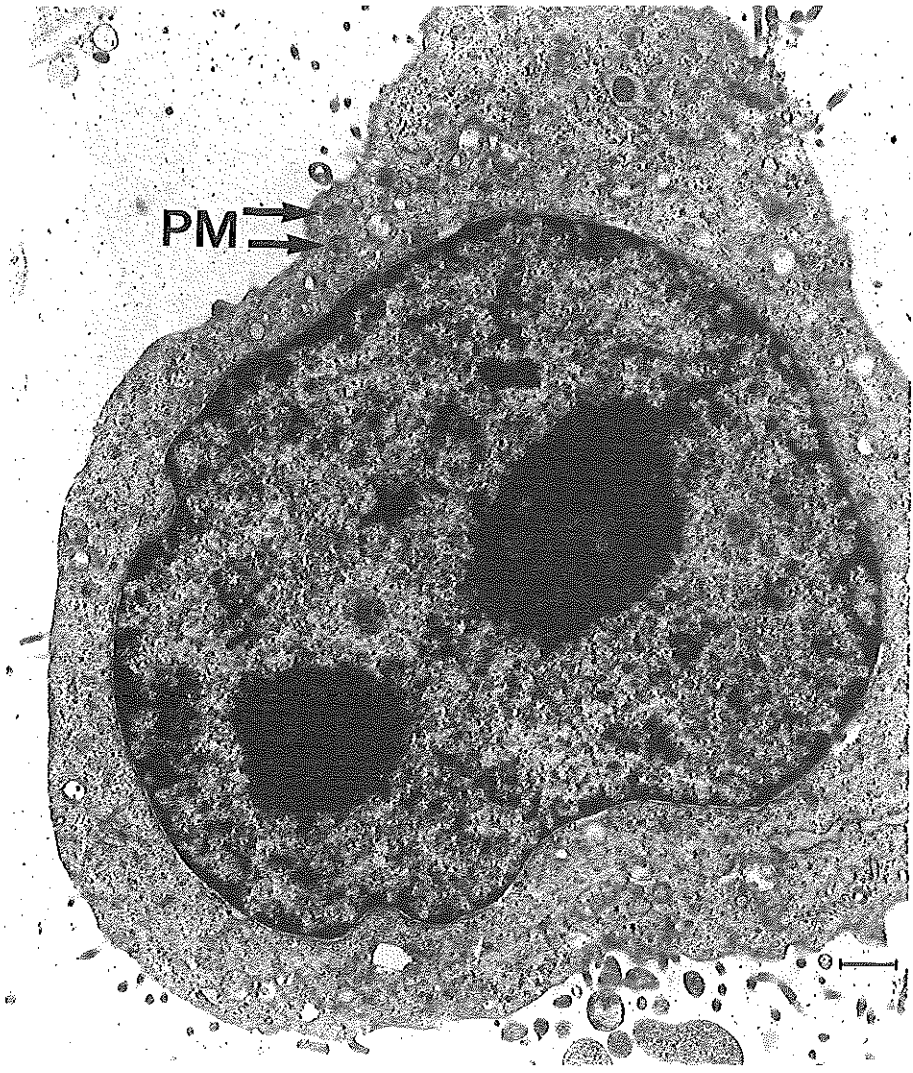
CELL LINE	ELECTRON MICROSCOPY	IMMUNOCYTOCHEMISTRY*					
	Melanosomes type	NKI/c3	HMB-45	S-100	HNK-1	BrdU	MCH class I expression on cell line
OCM-1[3]	I(II)	100[3]	100[3]	100[3]	0	n.d.	A24,A28
EOM-3	I	100 (p18)	100 (p18)	0 (p18)	100 (p18)	24 (p20)	-[†]
EOM-29	III, IV	100 (p5)	0 (p5)	0 (p5)	0 (p5)	<1 (p15)	A3,A30
OMM-1	III, IV (II)	100 (p26)	100 (p26)	100 (p26)	0.5[‡] (p27)	49 (p42)	A2,A11,B62
OMM-2	II, III, IV	100 (p17)	0 (p17)	0 (p17)	8 (p17)	<1 (p23)	A1
OMM-3	II, III	100 (p5)	0 (p5)	0 (p5)	4 (p5)	2 (p13)	A1,A11

* Percentage cells which bind the indicated antibody (passage number)
[3] Kan-Mitchell et al., 1989
† No detectable MHC class I expression
‡ Initially 50 percent of the cells expressed HNK-1 antigens.
Abbreviations: n.d., not done

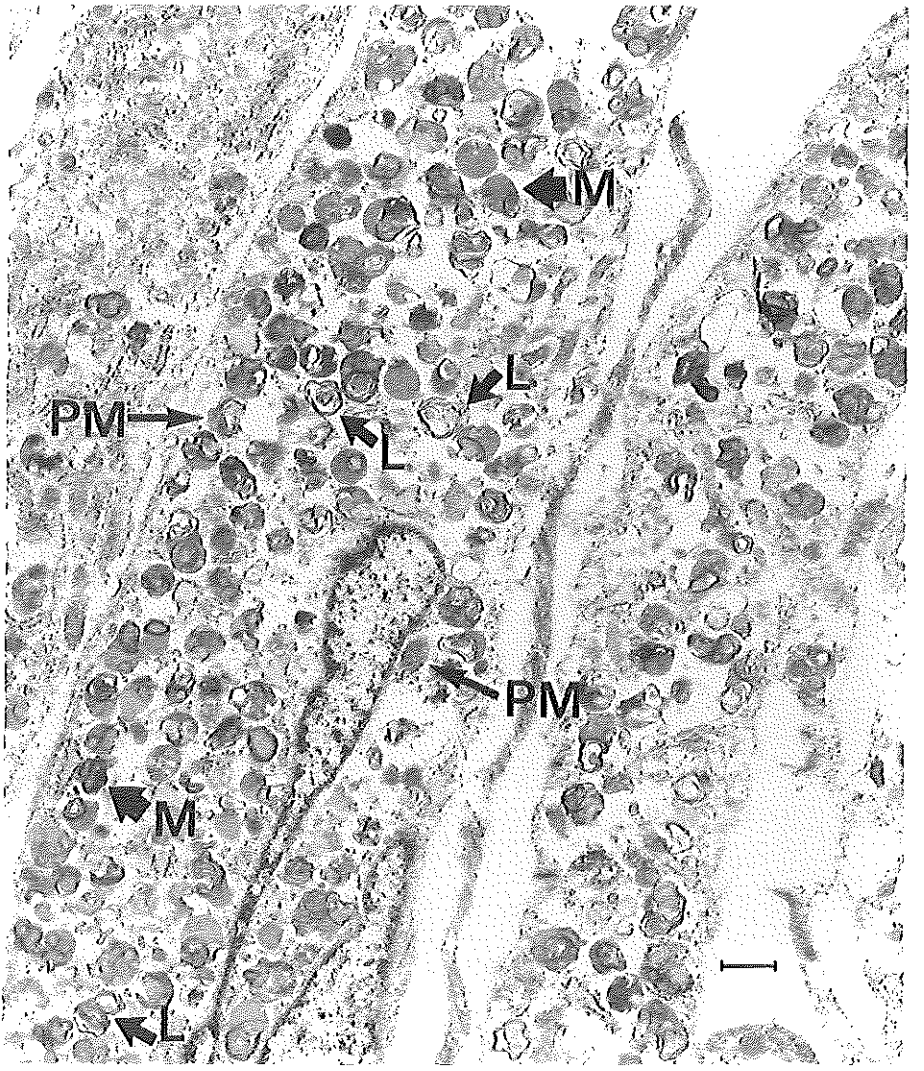
FIGURE 2 - Electron microscopy.



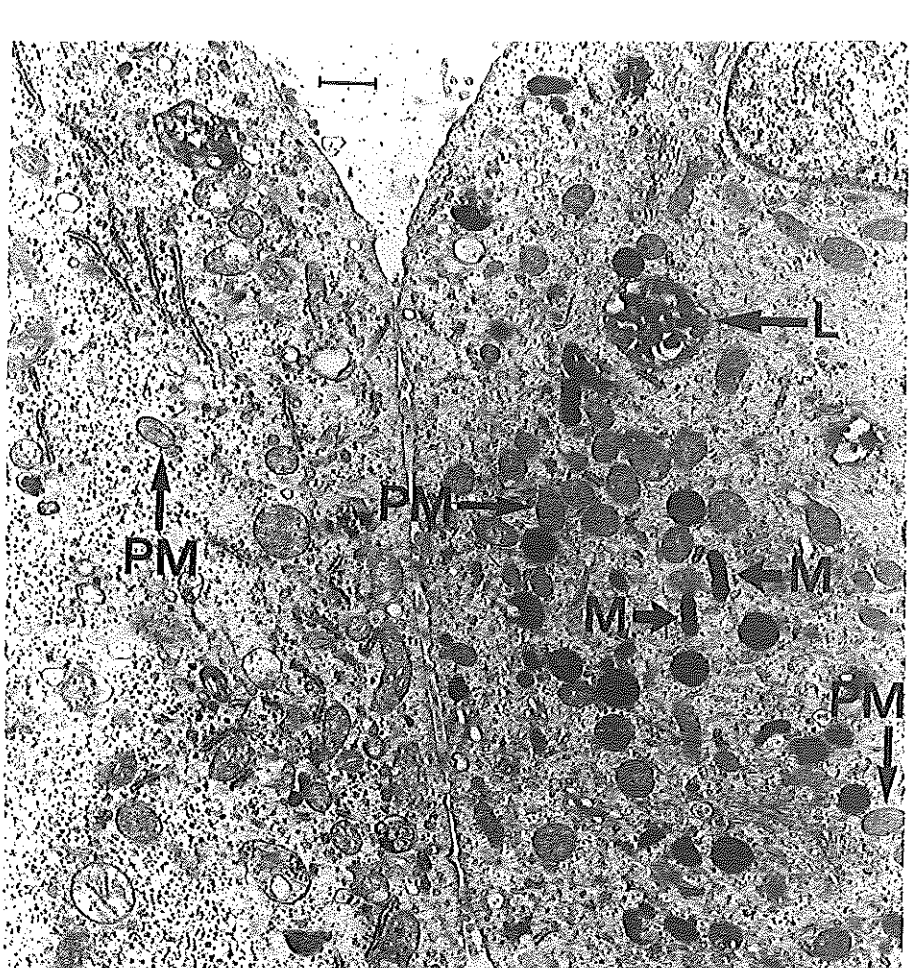
(A) OCM-1: part of an uveal melanoma cell. The cytoplasm contains a few melanosomes (M) and pre-melanosomes type I (PM) and sporadic type II (scale bar, .350 µm)



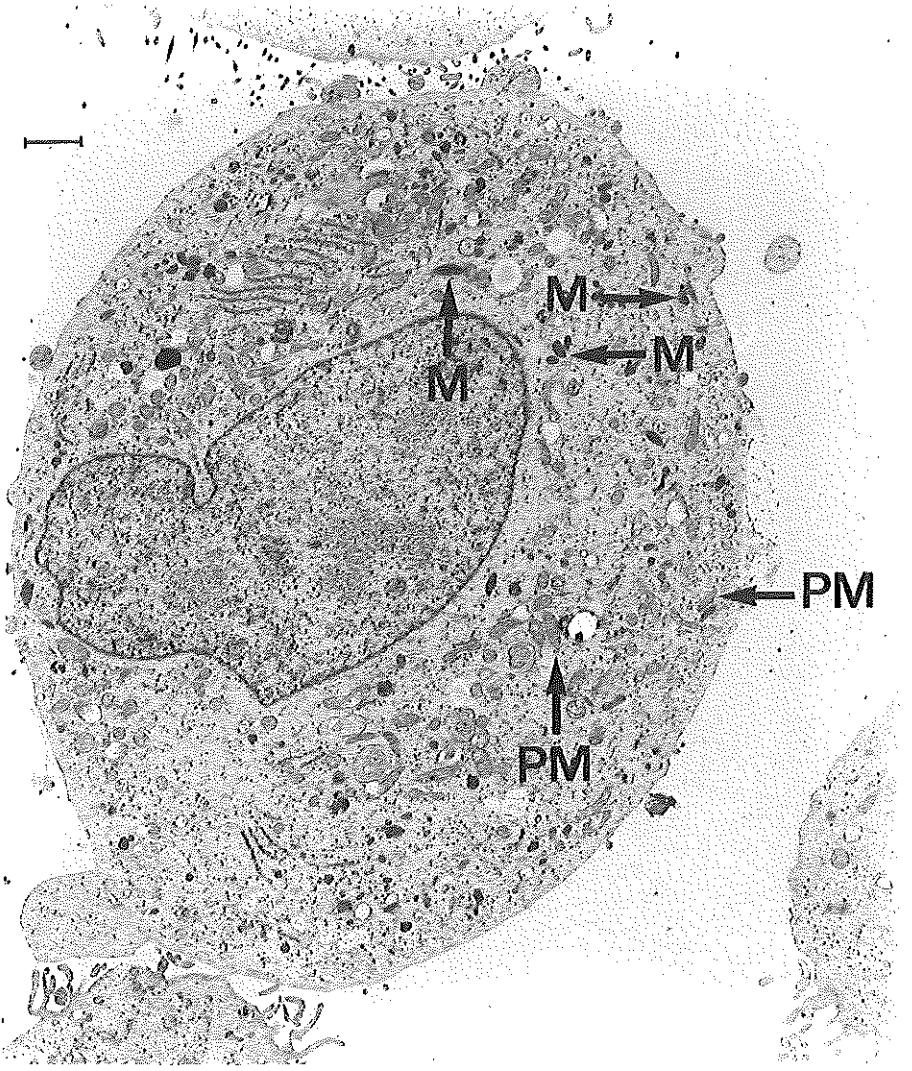
(B) EOM-3: very primitive, immature cell with a large nucleus and two prominent nucleoli. Only a few pre-melanosomes type I (PM) are present (scale bar, 1 μm)



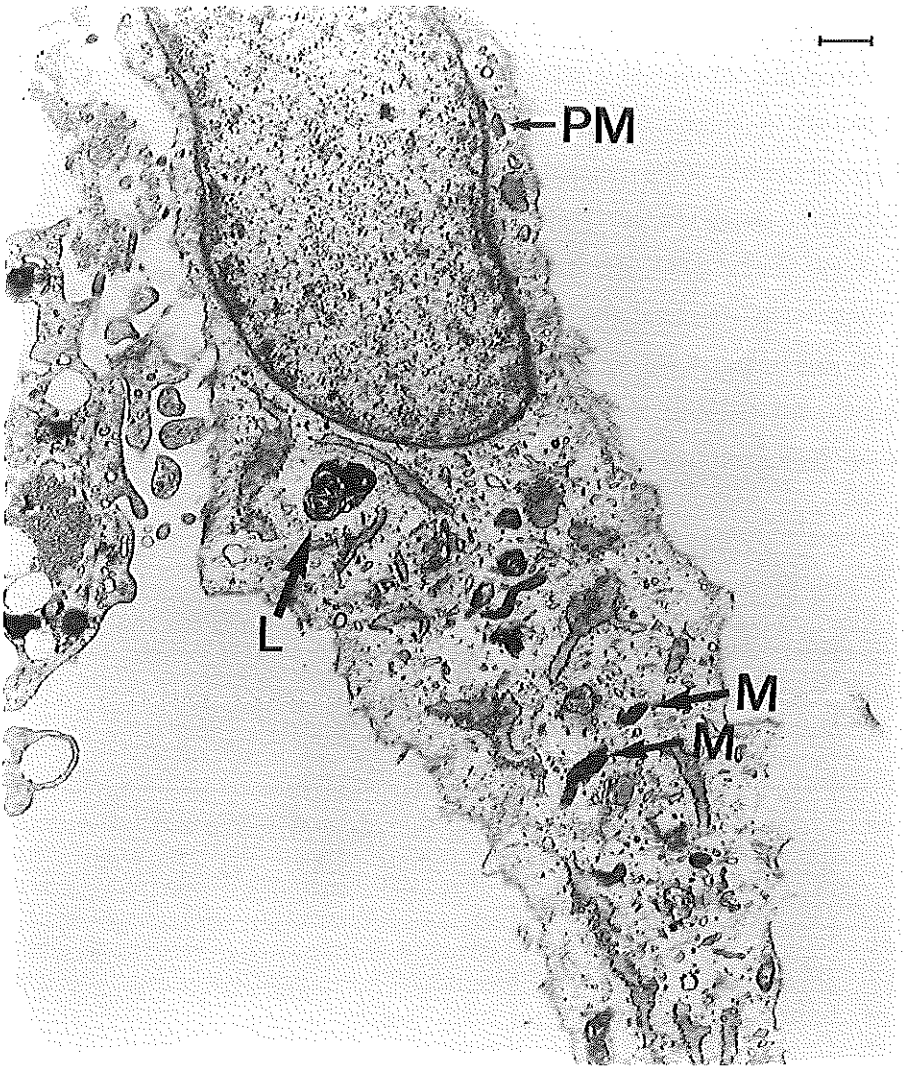
(C) EOM-29: heavily pigmented cells with abundant cytoplasm in which there are many melanosomes (M) and pre-melanosomes type III (PM) and lysosomal structures (L) (scale bar, 1 µm)



(D) OMM-1: two melanoma cells contain in the cytoplasm a large amount of osmiophylic (dense) melanosomes (M) and pre-melanosomes type III and IV, and only a few type II (PM). Note the presence of lysosomes (L) (scale bar, .583 µm)



(E) OMM-2: melanoma cell with an aberrant cytoplasm in which dense fusiform melanosomes (M) and pre-melanosomes (types II, III and IV) (PM) are observed (scale bar, 1 μm)



(F) OMM-3: spindle melanocytic cells. The cytoplasm contain large amount of rough endoplasmatic reticulum and pre-melanosomes types II and III (PM); a few lysosomes (L) are also present (scale bar, 1 μm).

Cytogenetic analysis

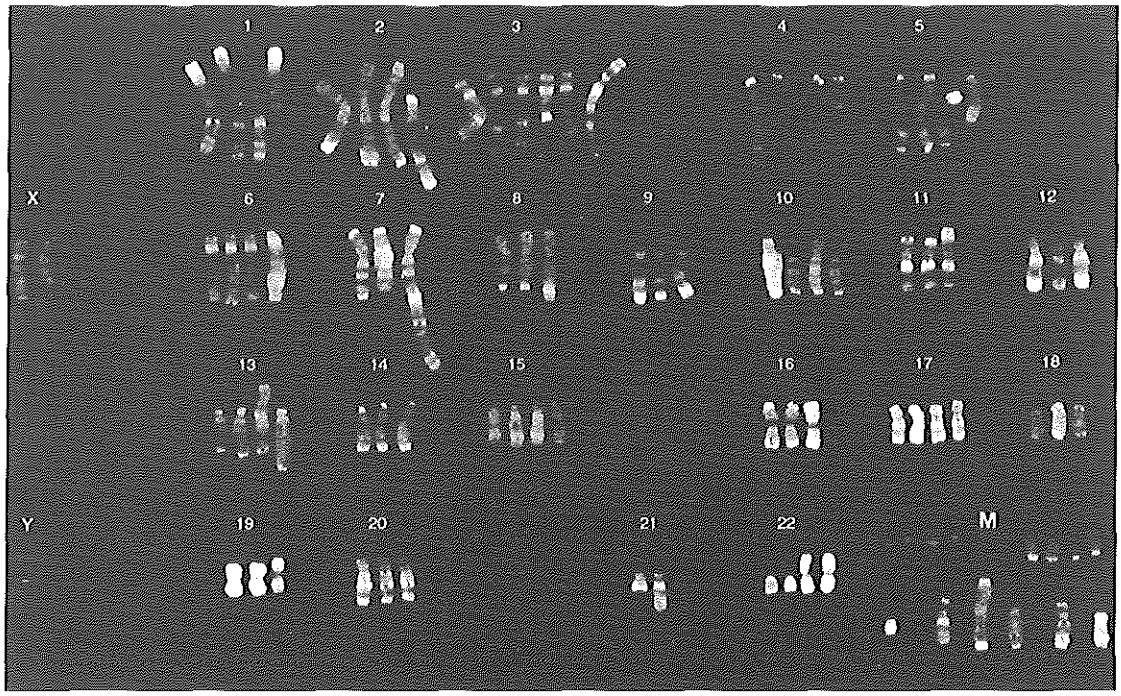
The karyotypes of the six cell lines are described in Table 3. The karyotypes were obtained from 10 to 25 metaphases from each cell line at different passages. Cells from cell line EOM-29 and OMM-3 displayed a normal karyotype. The stemline karyotype of OCM-1 was found to be tetraploid and to have many structural chromosomal aberrations (FIGURE 3A). These, among other changes, have resulted in a net loss of 3q and of the distal part of chromosome 8 (8q24-qter); monosomy 6 was observed in 60% of the metaphases of OCM-1. The stemline karyotype of EOM-3 was found to be pseudodiploid with numerical changes: a loss of Y, monosomy 6, trisomy 5 and 18 (FIGURE 3B). The stemline karyotype of cell line OMM-1 was near triploid with many structural and numerical changes, a net gain of chromosome 3, 7, 12 and 20, and loss of 4, 8p, 9, 11, 15, 17, 21, among others (FIGURE 3C). Cell line OMM-2 showed no clonal aberrations, but some non-clonal rearrangement, such as a monosomy 6 in 2 cells and an unbalanced translocation (14;22) in one cell.

TABLE 3

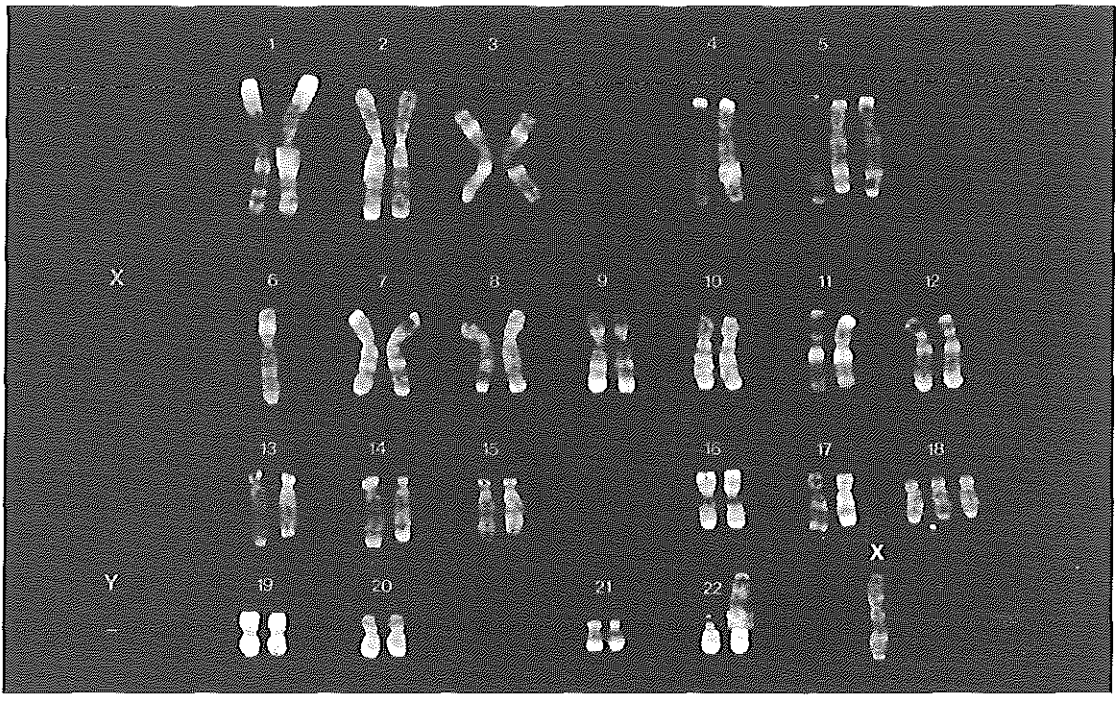
STAINING PATTERN IN THE METASTASES

CELL LINE	PASSAGE	NO OF METAPHASES ANALYZED	MODAL CHROMOSOMES	KARYOTYPE
OCM-1	>50	14	90-97	94,<4>,XX,-X,-X,del(1)(p21)[40%],der(2)t(1;2)(p31;q37),add(3)(q12), +del(3)(q13q23),del(4)(q12)[60%],-4[40%],add(5)(p11)[40%]),add(5)(q13), -6[60%],add(7)(q35),del(7)(q33),-7,add(8)(q24)x2,-9,-11,inv(11)(p15q24), del(12)(p12)x3,-12,add(13)(q31),-13or t(13q;14q)[60%],-15, add(16)(p13),-16[40%],-18[60%],-19[60%],add(20)(q13)x2 or x3,-20, add(21)(q21),-21,-21,del(22)(q11)x2,iso(22q)x2,+6-12 markers
EOM-3	10	22	46	46,X,-Y,+5,-6,+18
EOM-29	5	10	46	46,XY
OMM-1	8-10	25	60-68	64,<3>,X,-X,-Y,der(1)t(1;3)(p31;p13),der(2)t(2;5)(q32;q14?)x2, t(2;10)(q32;q25),+3[50%],-4,del(5)(q13q24),+7,add(8)(p11),-9,-11, +12,inv(13)(q12q34),-15,add(16)(p12),-17, del(19)(p12),+20,add(21)(p13),-21,+1-3 markers
OMM-2	11-14	19	46	46,XY[11]/44-45, with random loss of 1 or 2 chromosomes, [8 of which 2x -6]
OMM-3	1-4	21	46	46,XY

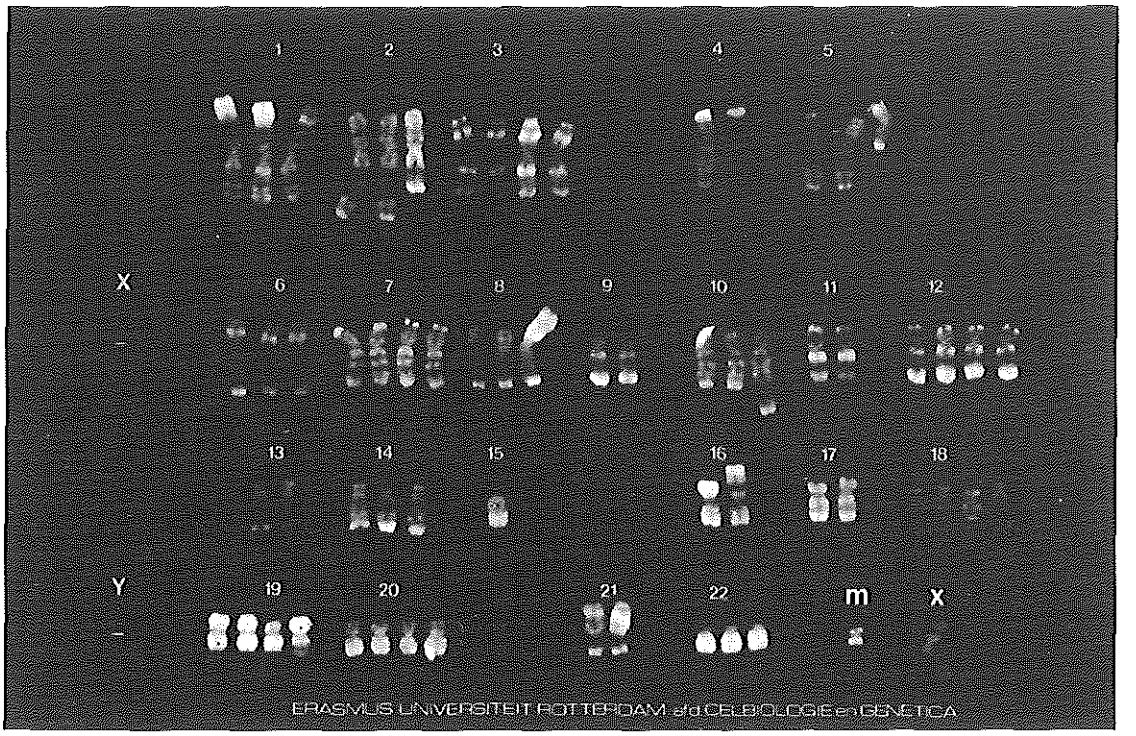
FIGURE 3 - Karyotypes of the cell lines.



(A) OCM-1



(B) EOM-3,



(C) OMM-1

DISCUSSION

Culturing of human uveal melanoma cells was first attempted in 1929 by Kirby,[14] but until the present date only a few cell lines from primary melanomas,3-6,15 and no cell lines from metastatic uveal melanomas have been described. The relative paucity of established uveal melanoma cell lines can be explained partly by the low incidence (1:100.000) of uveal melanoma and by difficulties in the culturing of primary uveal melanoma tissue.[1] Lack of therapeutic modalities for patients with hepatic metastases has led to a high mortality among them within a very short survival time, and hence provides a problem with respect to the availability of fresh metastatic tissue as well. In this study we were able to establish two primary uveal melanoma cell lines and three metastatic uveal melanoma cell lines. Although a limited overall number of tumours could be cultured at our institute, the success rate in the establishment of long-term

growing cell lines was relatively high for metastatic uveal melanomas (3/4) as compared with primary uveal melanoma (2/16). Metastatic lesions originate from selected cells that have proved to be capable of growing in secondary organs. This could be the reason for the high success rate in culturing metastatic cell lines. The present availability of primary and metastatic uveal melanoma cell lines make it possible to study the differences between primary and metastatic uveal melanoma in vitro.

To ascertain the melanocytic origin of the cultured cells, the cells were immunocytochemically studied by the use of melanoma-associated antibodies. S100, NKI/C3 and HMB-45 are known to be powerful markers for differentiating tumours of melanocytic lineage from other anaplastic tumours. In addition, electron-microscope studies revealed melanosomes and pre-melanosomes in all the cell lines described. The cell lines showed a considerable variation in passage time as measured by BrdU incorporation. The cell lines EOM-3 and OMM-1 had relatively high BrdU incorporation and were shown to have high growth rates with short passage times in culture. The cell lines EOM-29, OMM-2 and OMM-3 had low levels of BrdU incorporation and were also more difficult to maintain in culture. However, the cell lines OMM-2 and OMM-3 were stable over a long period of culture without showing any morphological changes. The cell line EOM-29 has been in culture for a relatively short period, showing no morphological changes, but immortality is not yet established. No difference in growth rate between primary and metastatic cell lines could be demonstrated by the BrdU incorporation.

Although epithelioid cells have often been regarded as the most malignant cell type, three out of six cell lines in our study, were of the spindle cell type (EOM-29, OMM-2, OMM-3). This in contrast to the histological cell type of the original tumour of the patient. This can probably be explained by the selective outgrowth of certain tumour cells in a favourable culture medium such that used by us. In case of the cell line EOM-3, the spindle cells of the fresh tumour culture were gradually overgrown by undifferentiated, anaplastic cells at passage five. After that time the uniform morphological cell type of EOM-3 remained unaltered. Whether the phenotypical change of the cell line EOM-3 is accompanied by genetical instabilities, reflecting one or more steps in tumour progression, or by simple selection is not known. Both cell

lines OCM-1 and OMM-1 consist of a heterogenous population of cells with a high growth rate, showing signs of selection of one of the morphological cell types present in the original tumour.

The normal expression of MHC class I molecules in five of the six cell lines indicates a possibility of a MHC class I restricted T-cell response in metastatic uveal melanoma patients. In the EOM-3 cell line, MHC-class-I expression is down-regulated and, as a result of this, increased susceptibility to natural-killer-cell activity has been demonstrated.[16] These data indicate that enhancement of the T-cell response by the use of allogenic irradiated cell lines might be one of the most promising ways to treat patients with metastatic disease.

In cytogenetical analyses of primary uveal melanoma, the most frequently described chromosomal abnormalities found are: monosomy of chromosome 3, net loss of genetic material of chromosome 6q and 8p, and gain of 6p and 8q.[17,18] It has been suggested that monosomy 3 and the isochromosome 8q are associated with ciliary-body melanoma and poor prognosis.[19] Cytogenetic analysis of our cell lines, showed a normal karyotype in three cell lines and karyotypic abnormalities in the other three, with partial loss of chromosome 6 among multiple other aberrations. OCM-1 and OMM-1 were near-tetraploid and triploid, respectively. In these cell lines, partial loss of 3q and 8p were seen, as a consequence of structural changes. In contrast to other results on cytogenetical abnormalities in ciliary-body melanoma, our cell line EOM-29, which was derived from a massive extrascleral growth of a ciliary-body melanoma, did not demonstrate the chromosome-3 and -8 abnormalities. We have found however no systematic differences in the karyotypes of the cultured cells of primary versus metastatic tumours. The karyotyping was assessed after establishment of the cell lines and thus, some chromosomal abnormalities could be the consequence of *in vitro* clonal selection and progression, instead of a representation of the situation of the tumour *in vivo*. In future studies, cytogenetic analysis of primary uveal melanoma cell cultures will hopefully provide more information on the karyotype of the tumour cells. Furthermore, complementary studies using molecular cytogenetic approaches and comparative genomic hybridization could give more detailed information on chromosomal abnormalities in fresh tumour and cell lines in culture.[20]

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CHAPTER 7

EXPRESSION OF *MAGE, GP100*, AND *tyrosinase* IN UVEAL MELANOMA CELL LINES

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Submitted for publication

ABSTRACT

Purpose: To evaluate the expression of the human genes *MAGE1, -2, -3, GP100* and *tyrosinase* in uveal melanoma.

Method: mRNA expression of *MAGE1, -2, -3, GP100* and *tyrosinase*, and the MHC class I expression were determined in five primary and three metastatic uveal melanoma cell lines.

Results: The expression of the examined genes was heterogenously in primary and metastatic cell lines. The cell lines OCM-1 and OMM-1 expressed *MAGE1, -2* and *-3*, whereas EOM-3, MEL202, 92-1and OMM-3 were negative. *GP100* was expressed in all cell lines, and *tyrosinase* in all but three (EOM-29, OMM-2 and OMM-3). Except for EOM-3, all cell lines expressed MHC class I molecules.

Conclusion: The tested melanoma-associated antigens can be expressed on uveal melanoma cell lines, as well as MHC class I molecules.

INTRODUCTION

Uveal melanoma is the most common intraocular malignancy in adults. Although several different therapies have been used for the local treatment of the primary tumor, life expectancy has not improved over the last two decades.[1] At least 50% of the patients develops metastases in distant organs, especially in the liver, which is eventually fatal within a few months.[2] New immunotherapy strategies using autologous or allogenic tumor cell vaccination are being developed for the treatment of cutaneous melanoma patients suffering from advanced metastatic disease.[3,4] Similarly these treatment modalities can possibly applied to metastatic uveal melanoma patients.

Immunohistochemical studies have shown that tumor-infiltrating lymphocytes (TIL) in uveal melanoma are mainly composed of helper (CD4+) and cytotoxic (CD8+) phenotypes.[5,6] From mixed lymphocyte/tumor cell cultures using TIL or

peripheral blood lymphocytes (PBL), cytotoxic T-cell (CTL) clones have been isolated which are able to recognize uveal melanoma cells in a major histocompatibility complex (MHC) class I restricted manner.[7] It has been shown that these CTLs recognize autologous as well as allogenic uveal melanoma cells and thus share the same tumor-associated antigens.[7] The anti-tumor response displayed by tumor-specific CTLs is the result of their ability to recognize "self"-proteins which are processed in the cytoplasm of tumor cells. Small peptides, processed from these proteins, are transported to the endoplasmatic reticulum, where they bind to newly synthesized MHC class I molecules. These MHC/peptide complexes are exported to the plasma membrane, where they can be recognized by specific CTLs.[8]

In recent years several cutaneous melanoma-specific antigens such as the *MAGE* family,[9-12] and melanoma-associated antigens (*GP100*, *tyrosinase*)[13-15] have been identified. *MAGE* genes are expressed in several malignancies but not in normal tissue, except for testis. These melanoma-specific antigens can be recognized by CTLs in the context of specific MHC class I molecules.[10] The melanoma-associated antigens, *GP100* and *tyrosinase*, are not melanoma specific since they are also expressed in normal melanocytes.[13-15] The *GP100* and *tyrosinase* antigens can be recognized by CTLs in an MHC class I restricted manner and can be recognized in the context of HLA-A2.

The purpose of the present study was to investigate whether uveal melanoma cell lines do express melanoma-specific and melanoma-associated antigens, such as *MAGE1, -2, -3, GP100* and *tyrosinase*. Since proper antigen presentation can only take place in the presence of a specific MHC class I molecule, the MHC class I expression was determined in these cell lines.

MATERIALS AND METHODS

Cell lines

At the department of Ophthalmology, Erasmus University Rotterdam, we succeeded in establishing cell lines from two primary uveal melanomas (EOM-3, EOM-29)[16,17] and from three subcutaneously localized metastases of uveal melanomas (OMM-1, OMM-2, OMM-3).[16-18] The other primary uveal melanoma cell lines used in this study were established and kindly provided by Dr. J. Kan-Mitchell, University of Southern California, San Diego, California (OCM-1),[7,16,19] Dr. B. Ksander, Schepens Eye Research Institute, Harvard Medical School, Boston (MEL202)[20] and Dr. I. de Waard-Siebinga, Department of Ophthalmology, University Hospital Leiden, The Netherlands (92-1)[21]. The cells were cultured until confluency, washed with phosphate-buffered saline (pH 7.4), and subsequently homogenized on ice with an ultra-turrax (TP 18-10) with intervals for 2 minutes. For each cell line, three flasks of 25 cm^2 (Falcon 3013E) were used.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The expression of the genes *MAGE1, -2, -3, GP100* and *tyrosinase* was determined by RT-PCR. RNA isolation was accomplished using the LiCl/ureum method, as described elsewhere.[22] For cDNA synthesis, 3 µg of total RNA was added to a 50 µl reaction mixture containing five times concentrated reverse-transcriptase buffer, 2.5 µg random primers (0.09 OD_{260} Units/µl) (Boehringer Mannheim), 0.5 µM DTT, 25 pM dNTP, and 20 units of RNAse inhibitor (GIBCO BRL). Incubation was performed for 1 hour at 37°C and subsequently for 10 min at 100°C. The thus produced cDNA could be stored at -20°C. The RT-PCR amplification of the cDNA was accomplished by the use of oligonucleotide primers, which are located in various exons of the *MAGE, tyrosinase, GP100* and human *βACTIN* genes. The RT-PCR of the *βACTIN* gene was used as a positive control. The sequences of these exons are: 5'-CGGCCGAAGGAA CCTGACCCAG-3' (CHO-14) and 5'-GCTGGAACCCTCACTGGGTTGCC-3' (CHO-12) for *MAGE1*;[11] 5'-AAGTAGGACCCGAGGCACTG-3' (CDS-9) and 5'-GAAGAGGAAGAAGCGGT

CTG-3' (CDS-7) for *MAGE2*;[11] 5'-TGGAGGACCAGAGGCCCCC-3' (AB1197) and 5'-GGACGATTATCAGGAGGCCTGC-3'(BLE5) (or AB-913, 5'GGAGTCCTCATAGGA TTGGCTCC3') for *MAGE3*;[12] 5'-TATTGAAAGTGCCGAGATCC-3' (1497/1516) and 5'-TGCAAGGACCACAGCCATC-3' (1839/1857) for *GP100*;[13] 5'TTGGCAG ATTGTCTGTAGCC-3' (HTYR1) and 5'-AGGCATTGTGCATGCTGCTT-3' (HTYR2) for *tyrosinase* gene;[23] 5'-GGCATCGTGATGGACTCCG-3' (exon 3 sense) and 5'-GCTGGAAGGTGGACAGCGA-3'(exon 6 anti-sense) for human *βACTIN*[8] (Pharmacia, Woerden, the Netherlands).

It was made sure that each polymerase chain reaction (PCR) contained 0.2 µl cDNA supplemented with 2.5 µl of 10x PCR Buffer (GIBCO BRL), 2.5 µl of 2.5 mM dNTP (Sphaero Q, the Netherlands), 5 µM of each primer, 0.5 units of Taq-polymerase (GIBCO-BRL) and water, to a total volume of 25 µl. In PCR-tubes (0.5 ml; Eppendorf safe lock) approximately 50 µl of mineral oil (Sigma) was added to the reaction volume to prevent evaporation. The cDNA was first denaturated in 5 min *(MAGE1,-2, GP100, βACTIN)* or in 4 min *(MAGE3, tyrosinase)* at 94°C. The amplifications were then induced; of *MAGE1* 36 cycles (1 min at 94°C, 3 min at 72°C), of *MAGE2* 36 cycles (1 min at 94°C, 2 min 67°C, 1 min 72°C), of *MAGE3* 30 cycles (1 min at 94°C, 2 min 72°C, 2 min at 72°C), of *βACTIN* gene 30 cycles (1 min at 94°C, 2 min 68°C, 2 min at 72°C), and of *tyrosinase* and *GP100* 36 cycles (1 min 94°C, 1 min 60°C, 1 min 72°C). A final incubation of 7 minutes *(MAGE1, GP100, tyrosinase)* or 15 min *(MAGE2,-3, βACTIN)* was performed at 72°C before the samples were cooled down to 4°C (Triblock, Biometra). A 15 ml aliquot of each reaction was size-fractionated on a 2% agarose gel stained with ethidium bromide. A Biorad Kb ladder was used as a calibration marker.

MHC Class I expression

To establish the expression of MHC class I molecules, the cell lines were tested in the complement-dependent microlymphocytotoxicity assay using a set of monoclonal antibodies directed against the various HLA-A and HLA-B antigens (monoclonal typing trays LM172 and LM272, One Lambda Inc., Canoga Park, CA, USA) Some of these monoclonal antibodies are reactive with only a single HLA antigen, whereas others recognize more than one. From the reaction pattern of the cells with these monoclonal antibodies the HLA antigen composition of the cells has been established.

RESULTS

The expression of the genes under examination in the various uveal melanoma cell lines is shown in TABLE 1 and FIGURE 1. These figures show that *MAGE1, -2,* and *-3* transcripts were found in the cell lines OCM-1 and OMM-1, while no expression of these genes could be demonstrated in the other cell lines. Expression of *GP100* was found in all examined cell lines. The *tyrosinase* transcript was found to be expressed in five out of the eight cell lines (not in EOM-29, OMM-2 and OMM-3).

The MHC class I expression of the uveal melanoma cell lines is also indicated in TABLE 1. The primary uveal melanoma cell lines OCM-1, EOM-29, MEL202 and 92-1 expressed MHC class I molecules; the latter two expressed HLA-A1 or HLA-A2 molecules. In the primary uveal melanoma cell line EOM-3, the MHC class I expression was down regulated and no immunocytochemical staining was detectable. Of the metastatic uveal melanoma cell lines, all were found to express HLA-A1 or HLA-A2.

TABLE 1

EXPRESSION OF MELANOMA-SPECIFIC AND -ASSOCIATED ANTIGENS, AND MHC CLASS I MOLECULES IN UVEAL MELANOMA CELL LINES

ANTIGENS	CELL LINES OCM-1*	EOM-3*	EOM-29*	Mel202*	92-1*	OMM-1†	OMM-2†	OMM-3†
MAGE-1	+	-	-	-	-	+	-	-
MAGE-2	+	-	-	-	-	+	-	-
MAGE-3	+	-	-	-	-	+	-	-
GP100	+	+	+	+	+	+	+	+
Tyrosinase	+	+	-	+	+	+	-	-
β-actin	+	+	+	+	+	+	+	-
MHC class I	A24,A28	no HLA	A3,A30	A1,A3	A2,A3	A2,A11,B62	A1	A1,A11

* primary uveal melanoma cell line;
† metastatic uveal melanoma cell line;
+ positive RT-PCR product; - no RT-PCR product

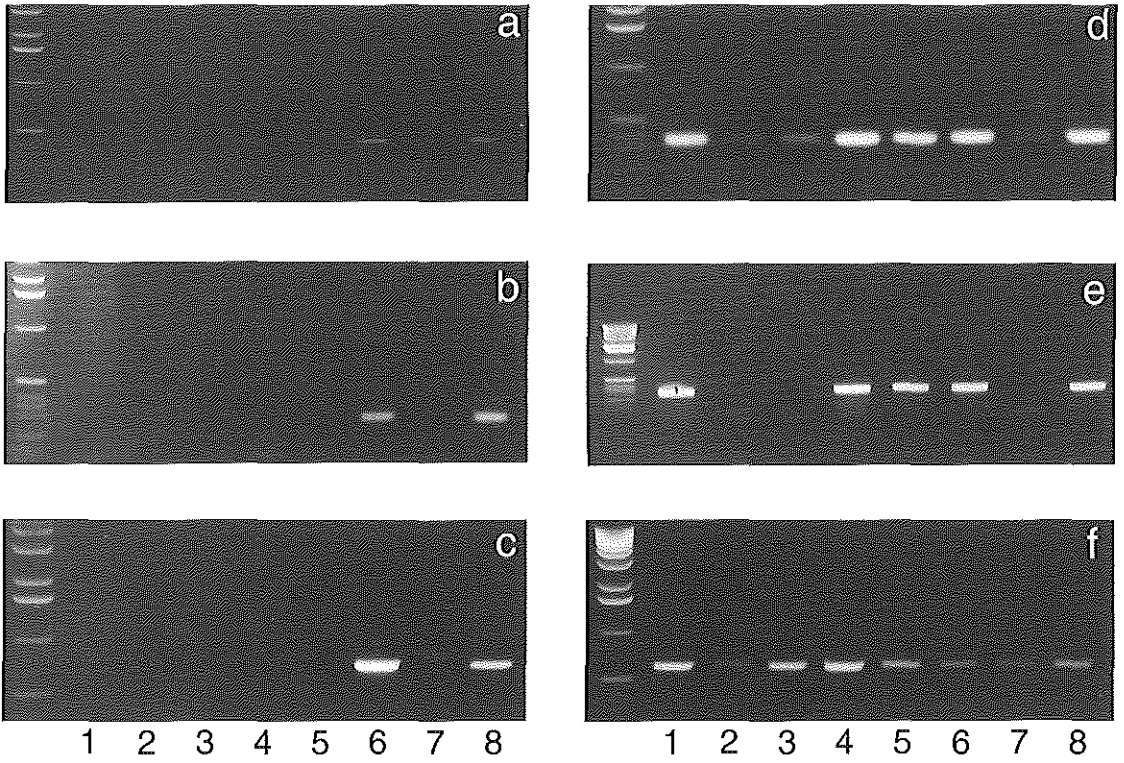

FIGURE 1. RT-PCR products of *MAGE-1* (a), -2 (b), -3 (c), *GP100* (d), *tyrosinase* (e), and *β-actin* (f) in human uveal melanoma cell lines; lane 1, EOM-3; lane 2, EOM29; lane 3, OMM-2; lane 4, Mel202; lane 5, 92.1; lane 6, OMM-1; lane 7, OMM-3 and lane 8, OCM-1.

DISCUSSION

For cutaneous melanoma patients with advanced metastatic disease new immunotherapeutic strategies are being developed. Patients are vaccinated with autologous tumor cells or allogenic cell lines transfected with the IL-2 or Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) genes.[3,4] Along the same line, a similar treatment modality can be deviced for patients suffering from uveal melanoma with distant metastases. In order to apply these new immunotherapeutic strategies for metastatic uveal melanoma patients, suitable uveal melanoma cell lines, which express melanoma-associated antigens and proper MHC class I molecules, are necessary. In the present study, we demonstrated that the melanoma-specific *MAGE1, -2* and *-3*

genes and the melanoma-associated *GP100* and *tyrosinase* genes can be expressed in uveal melanoma cell lines. Furthermore, MHC class I molecules can be expressed in these uveal melanoma cell lines.

In cutaneous melanoma, *MAGE1* is expressed in about 40% of the cases, *MAGE2* in 85% and *MAGE3* in 69%.[9-12] In our study, the mRNA transcripts of the *MAGE1, -2,* and *-3* gene are expressed in one of the primary (1:5) and in one of the metastatic (1:3) uveal melanoma cell lines. The higher expression rate of *MAGE2* and *MAGE3* found in cutaneous melanoma, could not be demonstrated in our uveal melanoma cell lines. The melanocytic-lineage specific antigen GP100 was found to be expressed in all uveal melanoma cell lines. The GP100 antigen was found to be recognized by the monoclonal antibodies NKI/beteb, HMB-45 and HMB-50.[24] These antibodies are highly sensitive markers for cells cutaneous as well as uveal melanomas and their metastases.[24,25] Recently it has been reported that the GP100 antigen is a type I transmembrane glycoprotein that is processed intracellularly and presented as peptides to CTLs in the context of HLA-A2.[14] Such CTLs, obtained from HLA-A2 positive melanoma patients, often display a broad reactivity not limited to melanoma cells only, but also to normal cutaneous melanocytes as well. Another antigen that is recognized by CTLs appeared to be the transcript of the *tyrosinase* gene. The *tyrosinase* gene is involved in the melanin synthesis and is expressed in all melanocytic-lineage specific cells; peptides derived from this protein can bind to HLA-A2 and HLA-A24 molecules.[23,26] Since HLA-A2 is expressed in about 49 % of the Caucasian population and *GP100* as well as the *tyrosinase* gene are expressed in a high rate of all uveal melanomas, it constitutes a useful target for specific immunotherapy. However, further studies are required to determine the side-effects of this type of therapy, such as the apparent destruction of choroidal melanocytes and retinal pigment epithelial cells.

The expression of the membrane-bound MHC/peptide complexes, recognized by CTLs, and of which the peptides are derived from the *MAGE, GP100* and *tyrosinase* genes were formerly described as being restricted to HLA-A1 or HLA-A2 molecules. However, more recent studies have elucidated that the complex binding of these peptides can probably also occur in other MHC class I molecules and is hence not restricted to HLA-A1 or HLA-A2 alone.[27] Considering this fact, it might therefore

be possible that CTLs can recognize MHC/peptide complexes of the OCM-1 cells, although they express HLA-A24 and HLA-A28.

In more than 90% of the uveal melanoma patients, liver metastases are the first signs of metastatic disease, with a median life-expectancy of only 2 to 7 months.[2] Therefore specific immunotherapy with transfected allogenic uveal melanoma cell lines might be a good approach. The cell lines OCM-1 and OMM-1 seem to be the best candidates to enhance immunoreactivity, because in these cell lines all melanoma-specific and melanoma-associated antigens, as well as the MHC class I molecules were found to be expressed. Additional studies on CTL responses to these uveal melanoma cell targets are necessary to demonstrate effective cytolysis.

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CHAPTER 8

RELATIONSHIP BETWEEN NATURAL KILLER CELL SUSCEPTIBILITY AND METASTASIS OF HUMAN UVEAL MELANOMA CELLS IN A MURINE MODEL

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ABSTRACT

Purpose: The purpose of this study was to determine the susceptibility of human uveal melanoma cells to in vitro and in vivo natural killer (NK) cell-mediated cytolysis and to determine if NK cells influence metastasis from the eye.

Methods: Four human uveal melanoma cell lines and one melanoma cell line derived from a metastatic lesion from a patient with uveal melanoma were tested for in vitro and in vivo NK cell-mediated lysis in a mouse model. Major histocompatibility complex (MHC) class I antigen expression was evaluated by flow cytometry. The role of NK cells in controlling the metastasis of uveal melanoma cells from the eye to the liver was examined in nude mice.

Results: Sensitivity to in vitro and in vivo lysis by human and murine NK cells was correlated with reduced expression of MHC class I antigens. Uveal melanoma lines expressing normal MHC class I antigen expression were insensitive to NK cell-mediated lysis, both in vitro and in vivo. Metastasis of uveal melanoma cells was inhibited by NK cell activity because disruption of in vivo NK function produced a sharp increase in the spontaneous metastasis of intraocular melanomas in nude mice.

Conclusions: There is considerable variation in the susceptibility of human melanomas to NK cell-mediated cytolysis. Susceptibility is closely correlated with reduced expression of MHC class I antigen expression. Disruption of NK cell function significantly increases the development of hepatic metastases from human uveal melanoma cells.

INTRODUCTION

Uveal melanoma is the most common primary intraocular tumor on adults.[1] Although primary uveal melanoma can be successfully treated by enucleation or radiotherapy, approximately 50% of uveal tumor patients will die from metastatic disease.[2] The liver is the most frequently affected organ in patients with metastatic melanoma from uveal

tract, and hepatic metastasis remains the leading cause of death in uveal melanoma patients.[3-5] Thus, preventing and treating hepatic metastases could have an important impact in the treatment of uveal melanoma patients.

In recent years, there has been a rekindled interest in the application of immunotherapy for a variety of tumors, especially cutaneous melanoma. Clinical trials involving the use of interleukin-2 (IL-2) and the adoptive transfer of tumor-infiltrating lymphocytes (TIL) have provided evidence attesting to the significant, albeit limited, efficacy of immunotherapy for metastases arising from cutaneous melanomas.[6-9] Although cutaneous melanoma has received considerable attention, only a limited number of studies have focused on the potential of immunotherapy of uveal melanoma. Ksander and co-workers have shown that antigen-specific cytolytic T lymphocyte (CTL) activity was demonstrable in TIL isolated from human choroidal melanomas.[10] Kan-Mitchell and co-workers were able to isolate and demonstrate melanoma-specific CTL from the peripheral blood of patients with choroidal melanoma.[11] Although cells with NK activity are present in TIL populations isolated from human choroidal melanomas,[10] no studies to date have examined the relative susceptibility of human uveal melanomas to NK cell-mediated cytolysis or the capacity of NK cells to prevent the metastatic spread of uveal melanoma cells. The present study was designed to address both of these crucial questions.

MATERIALS AND METHODS

Mice

Athymic nude BALB/c (H-2^d) mice were purchased from the Jackson laboratories (Bar Harbor, ME). The use of animals conformed to the ARVO Statement for the Use of Animals on Ophthalmic and Vision Research.

Tumor Cell Lines

Four human uveal melanoma cell lines, designated OCM-1, OCM-3, OCM-8, and EOM-3, were used. OCM-1, OCM-3, and OCM-8 were generously provided by June Kan-Mitchell (USC School of Medicine, Los Angeles, CA). OCM-1 is a predominantly spindle morphology while OCM-3 and OCM-8 are predominantly

epithelioid tumors.[11] OMM-1 was isolated from a subcutaneous metastasis in a patient with uveal melanoma patient and displays a mixed cell morphology.[12] The EOM-3 cell line displays an epithelioid morphology in vitro but was derived from a posterior choroidal melanoma of mixed cell morphology. OCM-1, OCM-3 and OCM-8 cells were cultured in HAM's F-12 medium containing 10% heat-inactivated fetal calf serum, 1% L-glutamine, 1% sodium pyruvate, 1% nonessential amino acids, 1% HEPES buffer, and 1% antibiotic-antimycotic solution. OMM-1 and EOM-3 cells were cultured in Dulbecco's modified Eagle's medium containing 10 % heat-inactivated fetal calf serum, 1% L-glutamine,1% sodium pyruvate, 1% vitamin solution, and 1% antibiotic-antimycotic solution.

Analysis of MHC Class I Expression

The expression of major histocompatibility complex (MHC) class I antigens was assessed by flow cytometry using a fluorescence-activated cell sorter (FACS) and was detected using a mouse monoclonal antibody directed against nonpolymorphic MHC class I determinants (IgG, clone BI-3d3; Accurate Chemical & Scientific Co., Westbury, NY). Mouse anti-H-2^k monoclonal antibody (IgG; clone 11-4.1; PharMingen, San Diego, CA) served as an isotype-matched negative control antibody. Single cell suspensions were prepared and washed in FACS buffer consisting of phosphate-buffered saline (PBS; Ph 7.4) with 1 % BSA and 0.02% sodium azide. Cells (10^6) were incubated with primary antibodies for 30 minutes on ice, washed three times, and then incubated with FITC-labeled secondary antibodies for 20 minutes at 0°C and washed an additional three times. Cell suspensions were fixed in 1% paraformaldehyde and assayed for positive staining on Epics Profile Analyzer (Coulter Eletronics Inc., Hialeah, FL). Gates were set at 1% of total cells, based on staining by secondary antibodies alone.

In vitro Natural Killer Cell Assay

Natural killer cell-mediated cytolysis was evaluated by a conventional 4-hour ^{51}Cr-release assay as described previously.[13] Human peripheral blood was used as a source of NK cells. Heparinized blood from healthy donors was layered onto

Histopaque-1119 (Sigma Chemical Co., St Louis, MO) and centrifuged for 30 minutes at 2,200 rpm at room temperature. The mononuclear cell layer was collected. BALB/c mouse spleens served as a source of murine NK cells. Spleen cells were pressed through sterile stainless steel screens, washed twice in HBSS, passed through sterile nylon mesh (Tetko, Elmsford, NY), and washed in HBSS an additional time. Total release counts per minute were determined by treatment target cells with 0.05 ml Hematall LA-Hgb Reagent (fisher Scientific, Pittburgh, PA) and spontaneous release by incubating target cells with complete medium in the absence of effector cells.

Liver Localization Assay

Assessment of hepatic NK cell-mediated immunity was performed by an in vitro clearance assay as described previously.[14] Briefly, ocular tumor cells were labeled in vitro with 100 µCi of $Na_2{}^{51}CrO_4$ for 1 hour at 37°C, washed three times with HBSS, and resuspended in HBSS at a concentration of 1×10^7 cell/ml. ^{51}Cr-labeled melanoma cells ($1x10^6$ in 0.1 ml) were injected into the lateral tail vein. Clearance of ^{51}Cr-labeled tumor cells was determined by counting the radioactivity (gamma emission) of livers, lungs, spleens, kidneys, and 0.5 ml blood removed from each animal 24 hours after intravenous tumor injection. Percent of radiolabel recovered was determined by dividing the counts per minute (cpm) for each liver divided by the total cpm recovered for liver, lungs, kidneys, spleen and 0.5 ml blood for each animal. The total cpm for each animal ranged from 35.000 to 45.000 cpm. In vivo NK cell-mediated elimination of radiolabeled tumor cells is reflected by a reduction in the radioactivity of the liver.[14]
In vivo NK cell mediated elimination of radiolabeled tumor cells is reflected by a reduction in the radioactivity of the liver.[14]

Intracameral Transplantation and Hepatic Metastasis Determination

A modified quantitative technique for the orthopic intracameral (IC) transplantion of precise numbers of tumor cells into the mouse eye has been described previously.[15] Mice were deeply anaesthetized with 0.66 mg of ketamine hydrochloride (Vetalar; Parke-Davis, Detroit, MI) given intramuscularly. Tumor cells (10^5/5µl) were inoculated IC using

a 1.0-ml Hamilton syringe fitted with a 35 gauge glass needle. Metastatic hepatic tumor foci were readily demonstrable by histopathological examination of the liver and were scored as previously described.[16,17] Severity of metastases was scored as: clear (0 = no discernible foci); minimal involvement (1^+ = metastatic tumors involved less than 10% of the liver); moderate (2^+ = metastatic tumors involved 10-25% of the liver); or extensive (3^+ = metastatic tumor mass involved ≥ 25% of the liver).

Cyclophosphamide Treatment

Cyclophosphamide (CY) was purchased from Mead Johnson Laboratories (Princeton, NJ) and dissolved in sterile HBSS. Panels of mice received intraperitoneal injections of CY (240 mg/kg) 24 hours before their sacrifice and use in the in vitro NK assays. For in vivo experiments, NK activity was inhibited by intraperitoneal injection of cyclophosphamide (240 mg/kg) on day 30 after tumor transplantation.

Poly I:C Treatment

Polycytidylic acid (poly I:C) was purchased from Sigma Chemical Co. (St louis, MO) and dissolved in sterile HBSS before treatment. Natural killer activity was stimulated in experimental animals by injecting poly I:C (100μg/mouse) intraperitoneal at weekly intervals after IC tumor transplantation and continuing until the time of necropsy.

Anti-asialo GM1 Treatment

Anti-asialo GM1 antibody was purchased from WAKO Chemical s(Dallas,TX) and used for in vivo depletion of NK cells. BALB/c nude mice were treated intravenously with anto-asialo GM1 (0.2 ml of a 1:10 dilution/mouse/injection) on day -3 and day -1 before the in vitro and vivo NK assays. For metastases experiments, anti-asialo GM1 antibody was injected once per week until the time of necropsy.

Statistics

Student's *t*-test was used to test the statistical significance of the data. Differences were considered significant when $P<0.05$.

RESULTS

Human uveal melanoma cells differ in their susceptibility to NK cell-mediated cytolysis

Although lymphoid cells bearing NK-associated cell membrane markers have been identified in uveal melanomas,[10] no studies to date have specifically examined the susceptibility of uveal melanoma cells to NK cell-mediated cytolysis. Therefore, five human uveal melanoma cell lines were examined for their susceptibility to in vitro cytolysis by murine and human NK cells. Two human uveal melanoma cells lines, OCM-3 and EOM-3, were susceptible to cytolysis by both murine (FIGURE 1). and human NK cells (data not shown). By contrast, the other three cell lines displayed insignificant susceptibility to NK cell-mediated lysis. Specific lysis by human NK cells was less than 10% for OCM-1, OCM-8, and OMM-1 (data not shown).

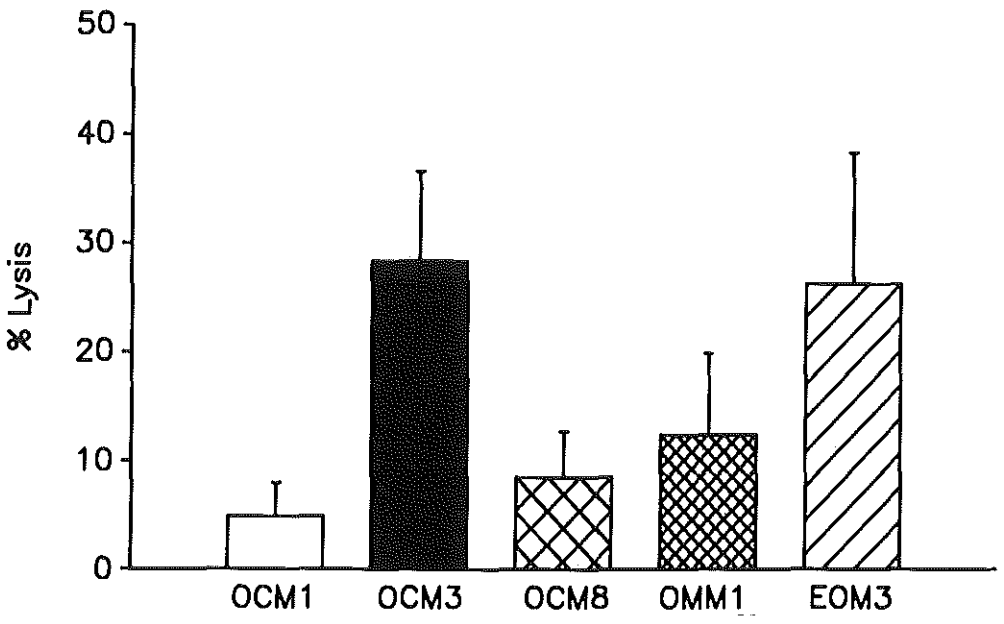

FIGURE 1. Susceptibility of five human uveal melanoma cell lines to cytolysis by murine NK cells. Cytolysis was measured in a conventional 4-hour ^{51}Cr-release assay using murine spleen cells at an effector-to-target ratio of 50:1. Results are expressed as mean ± standard deviation. NK, natural killer.

The susceptibility of human uveal melanoma cells to NK cell-mediated lysis in vivo was tested using a previously described clearance assay.[14] The elimination of intravenously injected radiolabeled tumor cells is a well-established method for evaluating NK cell-mediated lysis in vivo.[14] We have previously shown that human uveal melanoma cells preferentially localize in the liver following intravenous injection in nude mice.[18] Therefore, the in vivo elimination of human uveal melanoma cells was evaluated by determining the residual radioactivity of livers removed from nude mice 24 hours after intravenous injection of radiolabeled tumor cells. In some mice, NK function was stimulated by intraperitoneal injection of 100 μg of poly I:C 24 hour prior to the assay. In other groups of mice, NK cell activity was impaired by intraperitoneal injection of either cyclophosphamide (240 mg/kg) or anti-asialo GM1 antibody.

The in vivo clearance of intravenous injected uveal melanoma cells paralleled the in vitro results. That is, NK-sensitive OCM-3 and EOM-3 melanoma cells were eliminated more efficiently from the liver than were NK-insensitive OCM-1, OCM-8, and OMM-1 cells (TABLE 1).

TABLE 1

HEPATIC CLEARANCE OF RADIOLABELED HUMAN UVEAL MELANOMA CELLS

TUMOR	PERCENT RADIOLABEL RECOVERED* (P VALUE) CONTROL	POLY I:C	CYCLO-PHOSPHAMIDE	ANTI-ASIALO GM1
OCM-1	70.5	57.7 (.057)	80.3 (.18)	81.8 (.14)
OCM-3	64.2	52.3 (.089)	81.2 (.034)	83.7 (.02)
OCM-8	72.1	68.4 (.59)	76.2 (.54)	75.4 (.63)
OMM-1	67.5	64.3 (.61)	74.5 (.29)	73.6 (.37)
EOM-3	63.3	59.6 (.58)	79.4 (.035)	78.8 (.046)

* Percent dadiolabel recovered in the liver based on total counts recovered from lungs, liver, soleen, kidneys, and 0.5 ml of pherpheral blood. Probability (P) values for each group compered to untreated controls are shown in parenthese.

Moreover, disruption of in vivo NK cell function with either anti-asialo GM1 antibody or cyclophosphamide significantly impaired the liver clearance of OCM-3 and EOM-3 cells but had an insignificant effect on OCM-1, OCM-8, and OMM-1 cells. Thus OCM-3 and EOM-3 cells displayed significant in vitro and in vivo susceptibility to NK cell-mediated lysis.

NK sensitivity of human uveal melanoma is inversely correlated with MHC Class I antigen expression.

The NK sensitivity of murine fibrosarcomas and murine lymphomas has been shown to be inversely correlated with MHC class I antigen expression.[14-19] Accordingly, the expression of MHC class I antigens by human uveal melanoma cells was evaluated by flow cytometry. The results indicated that NK cell-mediated cytolysis was significantly reduced in human uveal melanoma cell lines I MHC antigens (FIGURE 2) By contrast, uveal melanoma cell lines less than 10 % positive for MHC class I expression were vulnerable to NK cell-mediated cytolysis (FIGURE 2). Thus, the NK sensitivity of human uveal melanoma is inversely correlated with expression of MHC class I antigens (TABLE 2.).[6]

TABLE 2

CORRELATION BETWEEN MHC CLASS I ANTIGEN EXPRESSION AND NK CELL SENSITIVITY

MELANOMA CELL LINE	MHC CLASS I EXPRESSION (% positive cells)	INVITRO NK CELL ACTIVITY (% Specific cytolysis)
OCM-1	90.12	3.5 ± 3.7
OCM-3	84.10	3.6 ± 4.6
OCM-8	76.85	5.9 ± 5.3
OMM-1	8.55	31.8 ± 9.8
EOM-3	4.71	35.2 ± 11.9

Abbreviations: MHC, major histcompatibility complex; NK, natural killer.

NK cells prevent the metastasis of uveal melanoma cells

The possibility that NK cells can limit or even prevent the metastasis of uveal melanoma cells was examined in mice. OCM-3 human uveal melanoma was selected for further study based on its in vitro and in vivo susceptibility to NK cell-mediated lysis. OCM-3 cells were transplanted into the anterior segments of BALB/c nude mice on day 0. In some mice, NK function was disrupted by either injecting cyclophosphamide intraperitoneally on day 30 or through weekly injections of anti-asialo GM1. Other groups of mice had their NK activity stimulated by weekly injections of the five untreated nude mice and none of the poly I:C mice (TABLE 3). However, 8 of the 10 nude mice with impaired NK cell activity harbored hepatic metastases of OCM-3 human uveal melanoma.

TABLE 3
INHIBITION OF UVEAL MELANOMS METASTASIS BY NATURAL KILLER CELLS

EXPERIMENTAL GROUP	SEVERITY OF METASTATIC FOCI					INCIDENCE
Untreated	0	0	2^+	0	0	1 / 5
Cyclophosphamide	2^+	2^+	0	3^+	3^+	4 / 5
Anti-asialo GM1	2^+	3^+	1^+	0	2^+	4 / 5
Poly I:C	0	0	0	0	0	0 / 5

Severity of metastasis based on relative size of metastaic foci detected by mocroscopic examination of histopathologic liver sections stained with hematoxylin and eosin, as described in Materials and Methods. All mice were necropsied on day 50.

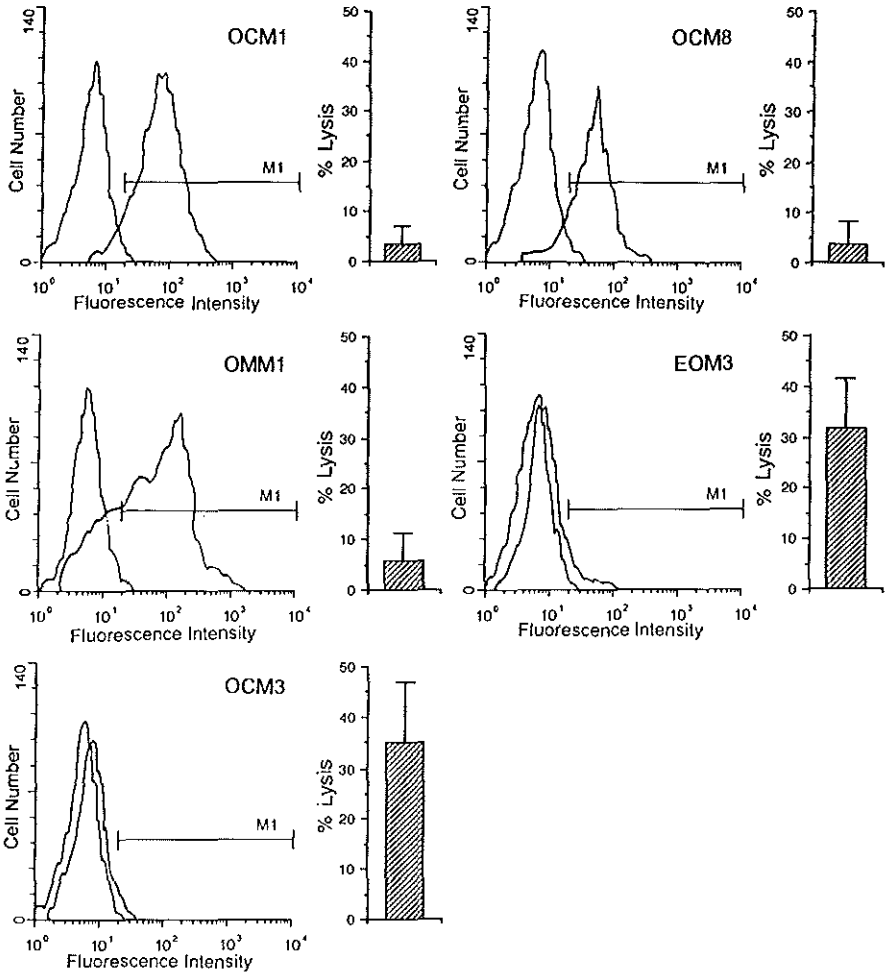

FIGURE 2. Inverse relationship between expression of MHC class I antigen expression and susceptibility of human uveal melanoma cell lines to NK cell-mediated cytolysis. Human peripheral blood served as a source of NK cells. For FACS profiles, the abscissa is alogarithmic scale that represents fluorescence intensity with specific antibody to MHC class I antigen. The ordinate is a linear scale representing the relative number of cells staining positively with the same antibody. The bar graphs represent the mean NK-mediated cytolysis (± standard deviation) of the respective target cell in a conventional 4-hour 51Cr-release assay using human peripheral blood lymphocytes as effector cells. Effector-to-target ratio was 50:1. MHC = major histocompatibility complex; NK = natural killer; FACS = fluorescence-activated cell sorter

DISCUSSION

Numerous studies have demonstrated that NK cells can limit the metastasis of experimental cutaneous B16 melanomas, and more recent studies have suggested that lymphokine-activated killer cells provide protection against metastasis in some patients with cutaneous melanoma.[6-9] However, to our knowledge, there are no published studies that have examined the susceptibility of human uveal melanoma cells to in vitro and in vivo NK cell-mediated cytolysis. Szalay and coworkers[20] have reported that impairment of NK activity promotes the metastasis of intracamerally transplanted B16 melanoma cells. Using the same murine model, these workers also reported that in vivo stimulation of NK cell activity with the biologic response modifier, LS2616, reduced the metastasis of intraocular B16 melanoma.[21] However, it should be pointed out that B16 melanoma is a murine cutaneous melanoma that may or may not accurately reflect the behavior of human uveal melanoma. As shown here and elsewhere, tumors of the same histologic origin can display vastly different susceptibilities to NK cell-mediated cytolysis, and one must exercise caution when extrapolating results from studies involving heterotopically transplanted tumor cells.

To study the role of NK cells in the prevention of metastases of intraocular melanomas in a prospective manner, a suitable animal model is required. We and others have transplanted B16F10 melanoma into the eyes of syngeneic C57BL/6 mice as a model of intraocular melanoma.[20-24] Greene hamster melanoma is perhaps the most widely used animal tumor in ocular melanoma research. However, like B16 melanoma, Greene melanoma arose as a cutaneous melanoma and therefore does not display the same metastatic behavior as uveal melanoma. Human uveal melanoma preferentially metastasizes to the liver,[3-5,25,26] whereas B16 melanoma characteristically spreads to the lungs.[25]

It is becoming increasingly clear that tumors transplanted to heterotopic sites often do not display metastatic behavior consistent with the original tumor.[27-31] Moreover many human tumors do not metastasize from heterotopic sites in nude mice but will form metastases after orthotopic transplantation.[27-31] The importance of orthotopic transplantation may be due, at least in part, to the influence of local organ-specific factors.[27] Thus, the importance of orthotopic, rather than heterotopic,

transplantation cannot be overemphasized.

The present model has several attributes that bear noting. Unlike murine B16 melanoma and Greene hamster melanoma, which are both cutaneous melanomas four of the five tumor lines were derived from patients with uveal melanoma. Thus, the intracameral transplantations in four of the five tumor cell lines was orthotopic, not heterotopic. Although the in vivo studies involved murine NK cells, it should be emphasized that the in vitro studies demonstrated that the NK cell-mediated cytolysis paralleled the results obtained with human NK cells. The orthotopically transplanted uveal melanomas used in this model demonstrate a strong predilection to metastasize to the liver and thereby mimic the human counterpart. By contrast, B16F10 melanomas invariably metastasize to the lungs after intravenous, intracameral, or subcutaneous transplantation.

The clearance of radiolabeled human uveal melanoma cells within the liver paralleled their in vitro sensitivity to NK cell-mediated cytolysis. Depletion of NK activity through the administration of either cyclophosphamide or anti-asialo GM1 significantly impaired in vivo clearance of radiolabeled uveal melanoma cells in the liver and resulted in more severe and extensive hepatic metastases. Although in vivo treatment with Poly I:C normally elevates NK activity, it failed to enhance the clearance of radiolabeled OCM-3 and EOM-3 cells within the liver and appeared to have an effect, albeit statistically insignificant, in enhancing the clearance of NK-insensitive OCM-1 cells. However, these minor aberrations in the vivo clearance in the Poly I:C experiments may be due to the unique qualities of hepatic NK cells in nude mice. Tzung and Cohen[32] have shown that the liver normally displays significantly greater activity than other organs because of the endogenous production of interferon-α/β by Kupffer cells. In nude mice, hepatic NK cell function appears to be elevated even further because liver clearance of radiolabeled tumor cells is 60% greater than of the euthymic counterparts.[33] Thus, our inability to enhance liver clearance of radiolabeled uveal melanoma cells in nude mice were already maximally activated, as suggested earlier. This proposition is consistent with the results from anti-asialo GM1 and cyclophosphamide treatments groups. Both of these agents are known to inhibit NK function in the liver of the nude mouse.[32,33] The striking impairment of liver clearance of radiolabeled uveal melanoma cells and the increased severity of hepatic

metastases in the cyclophosphamide and anti-asialo GM1 groups support the hypothesis that NK cells restrict uveal melanoma metastases.

In summary, the present findings indicate that human uveal melanomas differ in their susceptibility to in vitro and in vivo NK cell-mediated cytolysis. The degree of susceptibility to NK cell-mediated cytolysis is inversely correlated with expression of MHC class I antigen expression. That is decreased expression of class I antigens correlates with increased NK cell-mediated cytolysis. The results also indicate that disruption of in vivo NK activity can greatly increase the metastatic spread of uveal melanoma cells.

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CHAPTER 9

A CHICKEN EMBRYO MODEL TO STUDY THE GROWTH OF HUMAN UVEAL MELANOMA

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ABSTRACT

In vitro cultured human uveal or skin melanoma cells were injected into the chicken embryonal eye at a stage when the immune system was not yet mature. The melanoma cells were accepted as part of the organism by the host. Even single melanoma cells could be traced by morphological methods as well as immunohistochemical markers, such as S100, HMB-45, NKI/C3 and HNK-1. We found tumours in 10 and 40 per cent of the embryos injected with uveal melanoma and skin melanoma, respectively. The embryos did not exhibit abnormal development of the eye as a consequence of the microinjection and had a high survival rate (90 and 60%, respectively) during embryogenesis. With this model for uveal melanoma the growth and possible the metastatic behavior of uveal melanoma cells can be studied.

INTRODUCTION

The development of new techniques for the treatment of uveal melanoma has been hampered by the difficulties encountered in the culturing of uveal melanoma cells *in vitro* as well as the lack of animal models to study the pathogenesis. To date several cell lines of uveal melanoma have been described.[1,2] Albert and associates were able to culture human uveal melanoma tissue experimentally in nude mice by transplanting fresh (human) tumour tissue into the anterior chamber of the eye.[3] Kan-Mitchell and associates were able to culture uveal melanoma cells in rabbit eyes.[2] The xenografted animals, however, needed continous immunosuppressive therapy.

The purpose of the present study is to develop an animal model to study the growth of human melanoma cells in the chicken embryonal eye without using immunosuppressive agents. We injected *in vitro* cultured uveal or skin melanoma cells into the eyes of chicken embryos before maturation of the immune system. The injected cells were not rejected. We describe a simple model for studying the growth and metastatic behaviour of uveal melanoma; the advantages of this model are discussed.

MATERIALS AND METHODS

Embryos

Fertilized eggs of *Gallus gallus domesticus* were obtained from a local supplier and incubated in a forced draught incubator at 37°C and 60 % humidity. Embryos were staged according to the number of incubational or embryonal days (E). From E2.5 (embryonal day 2.5) onwards, the eggs were not tilted any more.

Microinjection

At E3.5, the embryonal eye is localized at the surface of the chorioallantoic membrane and easily recognized because the retinal pigment epithelium has just developed. The injection of melanoma cells into the eye with a microinjection system is therefore straightforward. After E3.5, the head of the chick moves farther away from the surface and dips towards the bottom of the egg so that *in vivo* injection of cells becomes technically more complicated. Microinjection was performed with a glass pipette with an internal diameter of 50 mm. Each microinjection represented about 10^2 - 10^3 single cells of either skin or uveal melanoma injected into the developing eye (1 - 10 ml injection).

Culture of skin melanoma

A human skin melanoma cell line derived from a lymph node metastasis was cultured in RPMI medium supplemented with 10 % heat-inactivated foetal calf serum, penicillin and streptomycin. This cell line was a fast growing monolayer culture, which underwent passage every week at 1:4 dilution and was grown continuously for more than two years. Confluent cell layers were trypsinized, washed with phosphate-buffered-saline (PBS), centrifuged, and the resulting pellet was suspended in PBS (5 X 10^6 cells/ml) for microinjection.

Culture of uveal melanoma

A 46-year-old patient who had undergone enucleation for uveal melanoma 28 years earlier had metastatic lesions in the liver, kidneys and subcutaneous tissue.

After obtaining informed consent, a subcutaneous metastatic nodule was excised when the patient was still alive and the specimen was immediately cultured. The metastatic tumours in liver, subcutaneous tissue and kidneys were consistent with metastatic amelanotic and melanotic melanoma of the mixed cell type (spindle and epithelioid cells). The patient died within 3 months of diagnosis after the clinical diagnosis of metastatic disease. At autopsy no skin melanomas or other primary tumours were found.

The tumour of the subcutaneous tissue was minced with a pair of fine scissors, irrigated continuously with Dulbecco's modified Eagle's medium and subsequently strained through cheese cloth. The resulting suspension consisted mostly of single cells and to a lesser extent small clumps of epithelioid cells. The tissue was characterized by a diversity of small and large multinuclear cells. Most nuclei contained multiple prominent nucleoli and numerous mitotic figures. The cells were washed twice with DMEM medium supplemented with 10 % heat-inactivated foetal calf serum, penicillin and streptomycin and seeded in a culture flask at 5 % CO_2. A feeder layer was not used.

The cultures, which consisted of adherent and non-adherent cells, were maintained in DMEM medium supplemented with 10% foetal calf serum. Confluent cultures underwent passage every 2 to 3 weeks at 1:4 dilution. The adherent cells were trypsinized, washed with PBS, centrifuged. The pellet suspended in PBS (5 X 10^6 cells/ml) for microinjection.

Antisera

S100,[4,5] NKI/C3,[6] HMB-45[7] and HNK-1[8] were used for the immunohistochemical studies of both the cell cultures and the histological sections of embryonal chicken eye. The S100 monoclonal antibody recognizes an acidic intracellular Ca^{++} binding protein and is a non-specific marker for melanoma cells.[9] Monoclonal antibodies NKI/C3 and HMB-45 are specific markers for precursors of melanocytes and melanoma cells. Monoclonal antibody HNK-1 recognizes a family of cell adhesion molecules (CD57), migrating neural crest cells and a series of neural crest derivatives.

Immunohistochemistry

The chicken embryo heads were decapitated at E10 and E19 and fixed in 10 percent formalin/PBS for at least two days, the eyes were enucleated, dehydrated and embedded in paraffin. The sections, 5-7 mm thick, were prepared by staining with haematoxylin/ eosin or by incubation with the primary antibody in a moist incubation chamber at room temperature for 30 minutes. Negative and positive controls for the primary antibodies were included. For immunoperoxidase staining, rabbit-anti-mouse peroxidase conjugated immunoglobulins were used as a second-step antibody. Endogenous peroxidases were inhibited by incubation for 25 minutes in a methanol/hydrogen peroxide (99:1 v/v) solution. Peroxidase was visualized with 0.1 % 3,3'diaminobenzidine HCL (Serva) and 0.01 % hydrogen peroxide. Sections were counterstained with haematoxylin/eosin for one minute. PBS with 0.1 % Tween-20 was used for all rinses. Cytocentrifuge preparations of cell cultures were analysed in a manner similar to that used for histological sections.

RESULTS

Culture of skin melanoma cells

The amelanotic cells grew as a uniform monolayer culture with several mitotic figures and possessed numerous lamellipodia.

Culture of metastatic uveal melanoma cells

One week after initial plating of the metastatic melanoma cells about 40% of the cells became adherent and nonadherent cells were removed weekly. After 3 weeks some adherent cells became rounded and had lost the ability to adhere. Some of the non-adherent cells (i.e. 25% approx.) were vital, according to trypan blue exclusion analysis, and hence multiplied in suspension, becoming adherent again after being seeded into new flasks. The culture was kept as a suspension culture and was grown continuously for nine months. To date there have not been any signs of differentiation, pigmentation or a decrease in the doubling time.

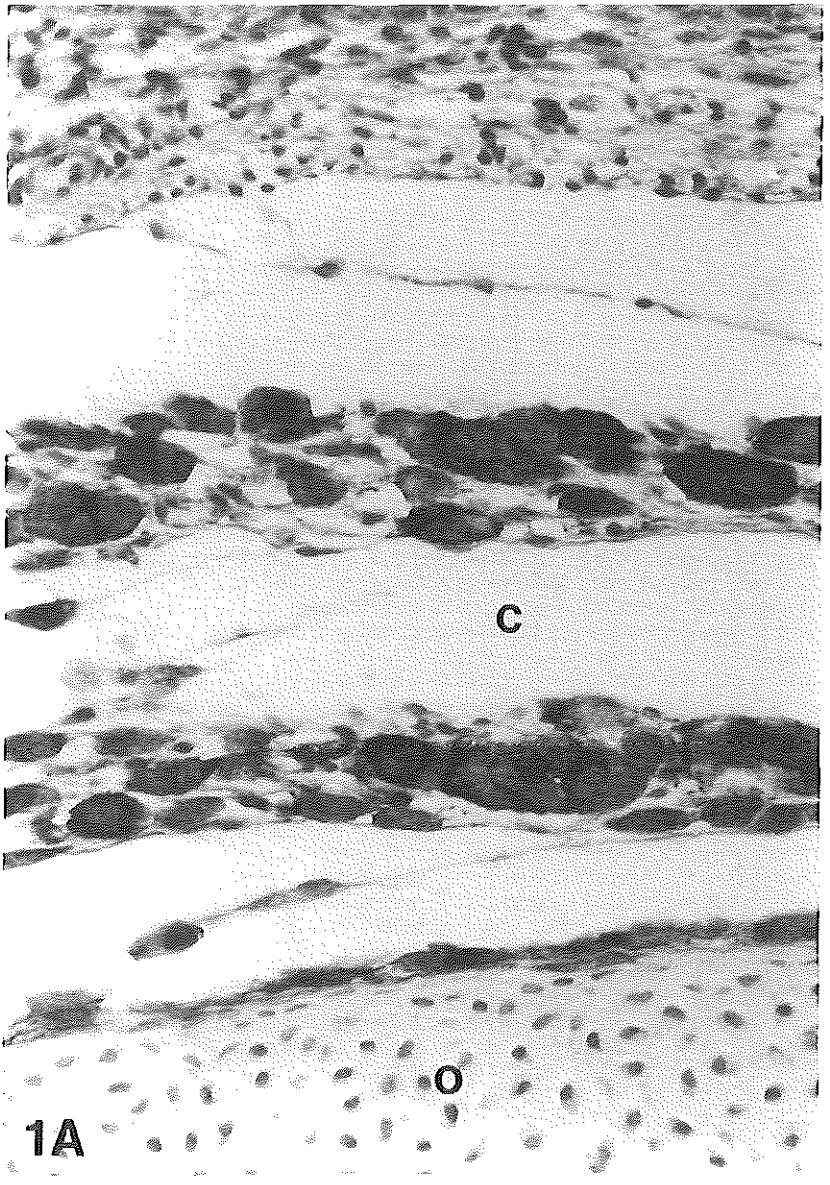
Microinjection of human skin melanoma cells

Twenty embryos were injected with skin melanoma cells; twelve embryos survived and eight of these embryos developed an intraocular melanoma. The eyes were removed for histological evaluation on days E10 or E19. The chicken eye was macroscopically normal. The injection site had undergone regeneration and could not be identified histologically. Histochemical studies revealed foci of melanoma cells, which appeared vital and mitotic figures were numerous. In all eyes, the melanoma cells grew on extra-cellular matrix structures in the choroid (FIGURE 1). There was no growth in the vitreous. At stage E10, large numbers of infiltrating cells were already present. The melanoma cells bound the monoclonal antibodies S100 and HNK-1 and the monoclonal antibodies HMB-45 and NKI/C3 were not bound (TABLE 1). Even single melanoma cells were easily detectable on days E10 and E19.

TABLE 1
EXPRESSION OF CELL MARKERS ON IN VITRO CULTURED AND XENOGRAFTED MELANOMA CELLS

	S-100	HMB-45	NKI/C3	HNK-1
Metastatic uveal melanoma	n.d.	++ (100%)	+++ (100%)	++ (100%)
Metastatic uveal melanoma cultured *in vitro*	++ (20%)	++ (70%)	++ (70%)	+++ (50%)
Uveal melanoma xenografted into chicken embryonal eye	+ (60%)	+++ (100%)	+ (70%)	n.d.
Skin melanoma cultured *in vitro*	+ (90%)	+ (100%)	+ (90%)	+ (100%)
Skin melanoma xenografted into chicken embryonal eye	++ (100%)	- (0%)	- (0%)	+ (100%)

n.d. = not determined; Intensity of staining: - = no staining; + = slight staining; ++ = intense staining; +++ = very intense (saturated) staining; percentage in paracenteses is the number of positive cells observed.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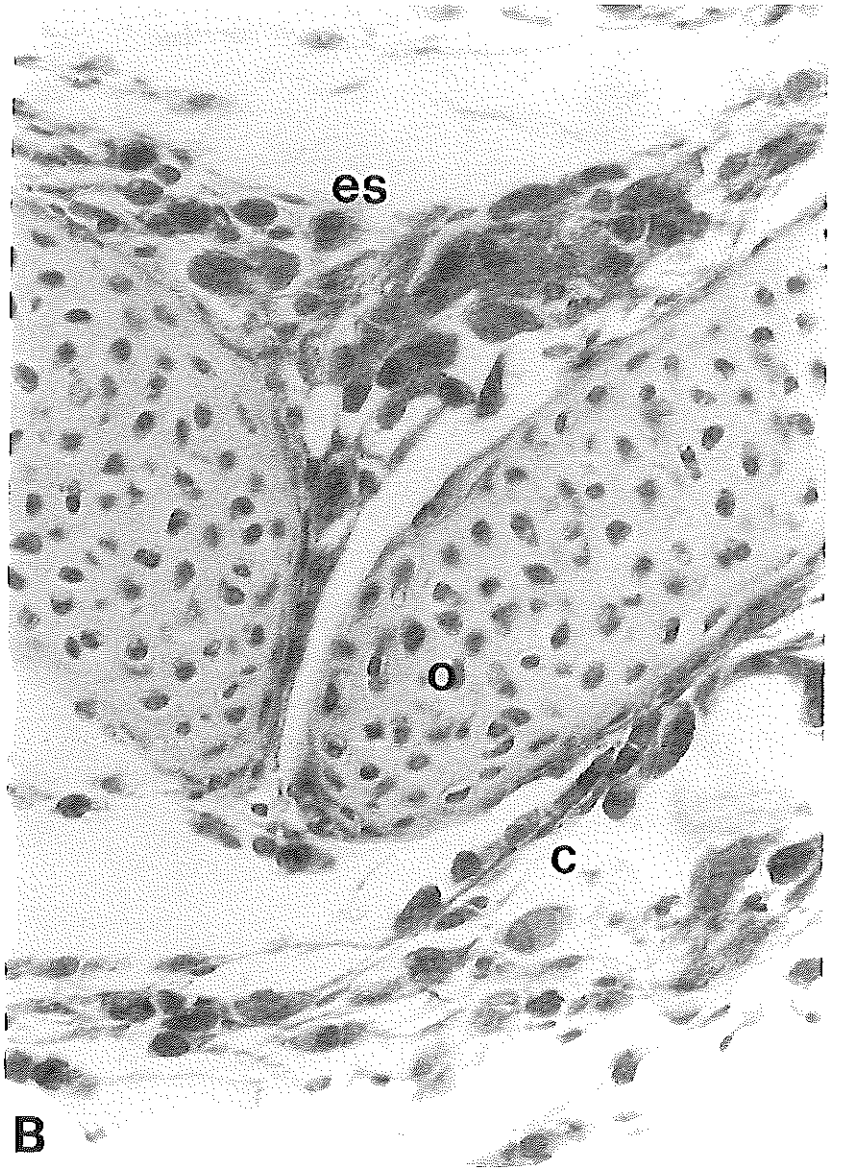

FIGURE 1.A and 1B. Growth of skin melanoma cells injected into the eye on E3.5 and analysed on E10, the tumour cells bind intensely the S100 antibody (a) and E19 (b). Infiltration of melanoma cells are observed in the choroid (c), between the cartilage ossicles (o), and extra-sclerally (es). (Magnification 320, 420x, respectively)

Microinjection of human metastatic uveal melanoma cells

Eighteen out of twenty injected embryos survived with two embryos (E19) exhibited intra-ocular tumour growth (FIGURE 2). On histological examination the chicken eye had developed normally and the injection site could not be recognized. Clones of metastatic uveal melanoma cells were seen adherent to the ciliary body and along the hyaloid artery which is very pronounced in the chicken embryo compared to human embryos. The tumor nodules derived from uveal melanoma were smaller than those originating from cutaneous melanoma.

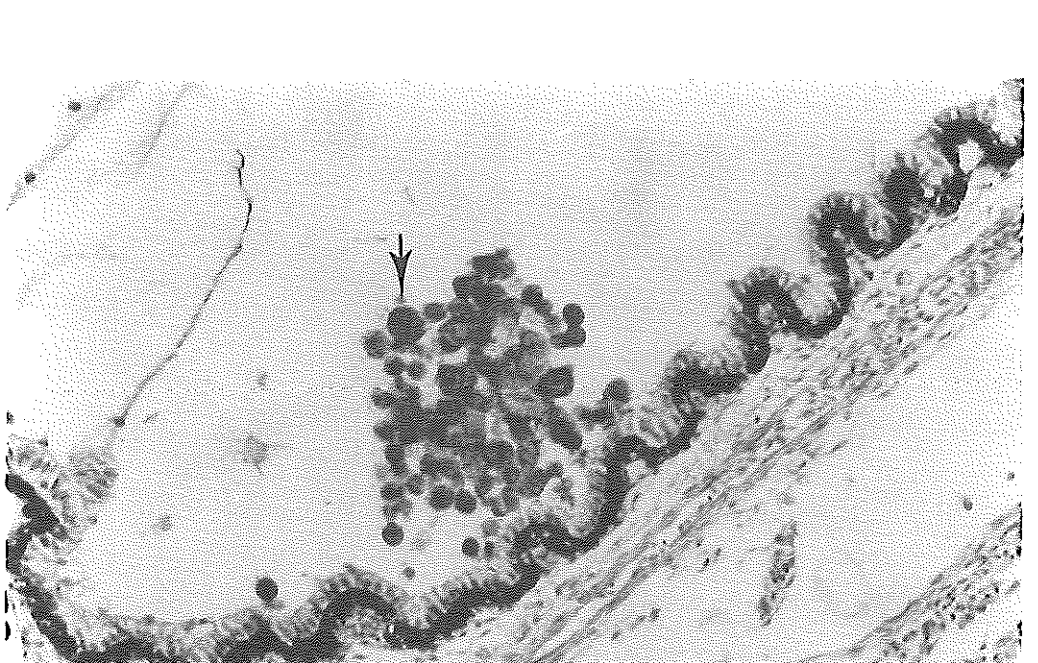

FIGURE 2. Growth of uveal human melanoma cells along the ciliary body in an E19 eye. The tumour cells stain intensely with the HMB-45 marker (arrow). (Magnification 750x)

Immunohistochemistry

We determined the expression of four markers (S100, HMB-45, NKI/C3, and HNK-1) on skin and uveal melanoma cells from the patient, on *in vitro* cultured cells and on the xenografted melanoma cells (TABLE 1). Some examples are illustrated in FIGURE 3. For both skin and uveal melanoma we found a difference in marker expression which depended on the growth system used. Skin melanoma cells in particular lost all marker expression for HMB-45 and NKI/C3 when xenografted into the chicken.

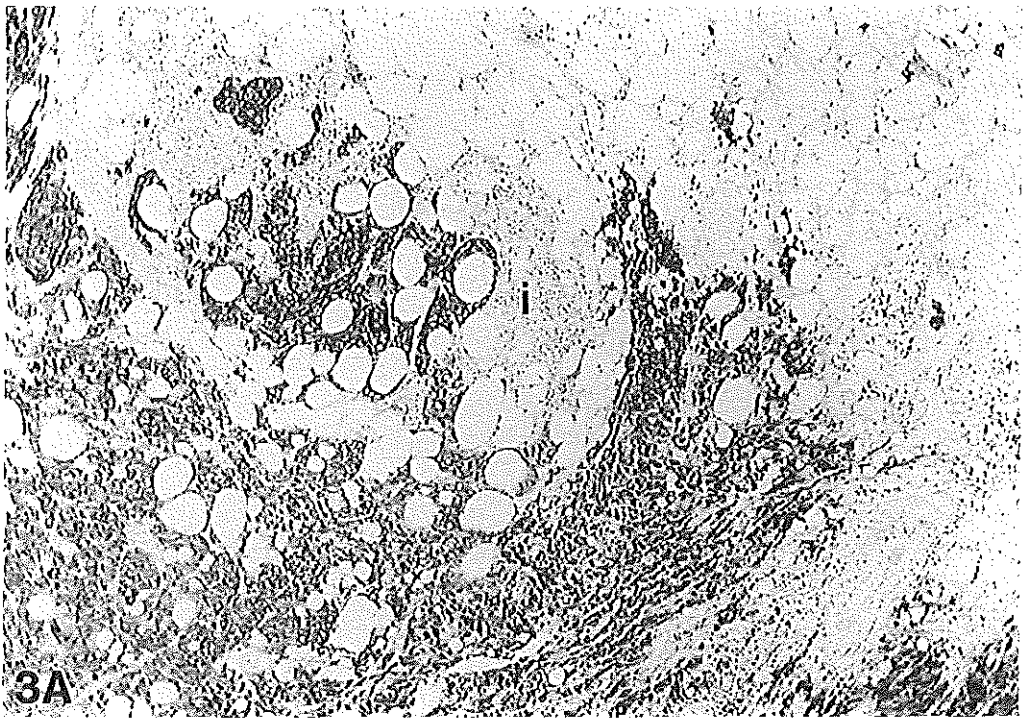

FIGURE 3A. Human uveal melanoma metastasis in subcutaneous tissue stained with HMB-45 uveal melanoma (u) infiltrating lymphocytes (i) (Magnification 150x)

In contrast to skin melanoma, xenografted human uveal melanoma retained the marker expression for HMB-45 and NKI/C3. The S100 marker revealed no large differences in marker expression for either type of melanoma irrespective of growth conditions. We conclude from the results summarized in TABLE 1 that xenografted human uveal melanoma cells can be traced on histological sections of the chicken eye with the four markers and that, although the expression differed depending on the microenvironment, marked change in morphology of the xenografted uveal tumour cells does not occur.

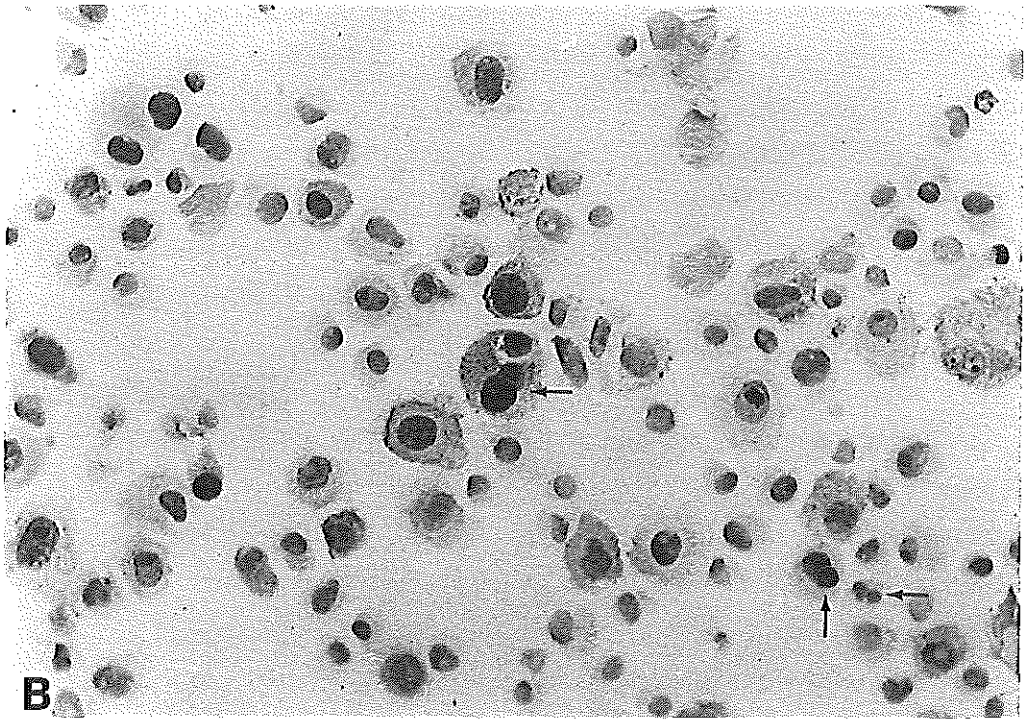

FIGURE 3B. Cytocentrifuge preparations of uveal melanoma cells stained with HNK-1. About 50% of cells are positive, a diversity of cell types can be seen. Several cells are multinucleated (arrow). (Magnification 460x)

DISCUSSION

The prognosis of primary uveal melanoma has not improved despite recent introduced irradiation techniques.[10-12] It is assumed that the presence of clinical disease and micro-metastasis at the time of treatment of the primary tumour is the main cause of the low survival rates.[13] Uveal melanomas grow slowly compared to cutaneous melanomas, metastasis is mostly observed 5 year after the clinical observation of the primary tumour. After the clinical detection of the metastatic disease, in general, patients die within a year,[14,15] including in the majority of cases liver involvement.[16]

The paucity of animal models for human uveal melanoma has limited progress in our understanding of the aetiology, tumourigenesis, tumour progression, and eventual treatment. Since knowledge about the growth and metastasis of uveal melanoma is limited, the development of efficient diagnostic and therapeutical modalities is stagnating. Until recently it was difficult to culture primary and metastatic uveal melanoma *in vitro*. Albert and associates used a feeder layer and epidermal growth factor to support cell attachment and growth.[1] The use of epidermal growth factor in combination with cholera toxin had a synergistic effect since contaminating cells were inhibited and proliferation of the melanocytes was stimulated. Kan-Mitchell and associates did not use these additives in order to select the most aggressive of the growing melanoma cells as the poor culture medium was sufficient to select aggressive cells.[2]

We cultured cells of a metastatic uveal melanoma containing fast-growing epithelioid cells with many multinucleated cells. Except for a different mincing procedure and the use of DMEM medium instead of RPMI, the culturing procedure was the same as that described by Kan-Mitchell and associates.[2] We obtained a cell culture which contained heterogeneous morphological cell types. We were able to culture the metastatic uveal melanoma cells for at least 9 months. Seventy percent of the cells were positive for two melanoma markers (HMB-45 and NKI/C3) and 50 percent were positive for HNK-1.

The injection of primary tumour cells into the anterior chamber and vitreous cavity of nude mice is described but no follow-up studies have been published.[3] Recently Kan-Mitchell and associates described a rabbit model that was suitable for

studying the growth of uveal melanoma, in which uveal melanoma was grown in the rabbit anterior chamber.[2] A disadvantage of this animal model is the daily injection of cumulative doses of an immunosuppressive agent.

We describe a new model for studying the growth of human uveal melanoma cells, which uses the chicken embryo. After culturing, either skin or uveal melanoma cells were injected into the developing eye of chicken embryos before maturation of the immune system. Both melanoma cell types grew in the developing embryonal eye. Skin melanoma cells grew much faster in culture and in the chicken embryonal eye than uveal melanoma cells. The observed foci of both tumour types were alive; mitotic figures were frequently found implying active growth. The behaviour of the tumour cells in the chicken eye was rather consistent. The tumour cells grew along the tunica vasculosa of the lens, in the choroid and along the sclera. Apparently, these cells needed a matrix to start growth. At E19 large infiltrating tumours of skin melanoma were observed. Probably due to the slower growth of uveal melanoma, comparable infiltrations of uveal melanoma cells were not found at E19. In general the uveal melanoma cell culture grew much more slowly in the embryos and a much lower percentage of embryos had histologically detectable uveal tumours.

Both, uveal and cutaneous tumour cells, could be recognized easily on histological sections of the chicken embryonal eye by means of their morphology and by antibody markers. Separate tumour cells could be readily traced. In this study the skin melanomas could be traced with S100 and uveal melanoma cells with the melanoma marker HMB-45 and to a lesser extent with NKI/C3 and S100. There was a difference in expression of the markers between the observed tumour foci and the injected cells, indicating that clonal selection had occurred *in vivo* resulting in a different marker make up. Another possibility is that the altered marker expression is environment-dependent.

In addition to the potential to study the growth and metastasis of human uveal melanoma cells in an environment that tolerates xenografted cells, this model has several advantages: a) low cost b) large series can be studied c) easy technique d) high survival rate for treated embryos e) the possibility of screening therapeutical agents. Another advantage is that funduscopy in hatched chicken and adult chicken can be performed. We hope this experimental model will contribute to the understanding of the tumourigenesis of uveal melanoma and the metastasis of this neoplasm.

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CHAPTER 10

DISCUSSION AND FUTURE PERSPECTIVES

In this final Chapter we will turn back to the basic questions that formed the background to the studies presented in Chapters 2 to 9. We will discuss what these studies have revealed concerning these questions, and propose what direction the investigations should take next in order to arrive at a therapeutically more successful approach of uveal melanoma. As was explained in section 1.2, the present thesis set out to investigate some of the key elements involved in the metastatic process of this type of cancer, with the aim of providing insight in how to treat uveal melanoma-related metastatic disease as promptly as possible in order to decrease the overall mortality rate of 50 %. The three basic points of interest were directed at the origins of the metastases, differences between primary and metastatic uveal melanoma, and the treatment (and prevention) of metastatic disease (see 1.2).

Firstly, we will take another look at the subject of enucleation versus radiotherapy of the primary tumor-containing eye, that was introduced in section 1.1.2 (Chapter 1). As was indicated there, the relative indication for enucleation or radiotherapy is not directed by the relative risk for metastases but by tumor control. Preliminary results do not show a difference in survival between either of these therapies. A possible demonstrated difference in survival between patients treated by either one of the therapies cannot be expected within 6 years after initial treatment (Manschot, 1992). The COMS investigation will study this problem in a large group of patients with medium and large sized tumors. However, among these patients specifically there is a relatively high mortality rate, and the majority of them is probably not curable by applying any type of local treatment. In this context the question rises whether not patients with a small primary tumor, who in most cases are potentially curable, should be included in such a study. Apparently, regarding the ascertaining of the safety of conservative treatment modalities, especially long-term studies of cases of small primary tumors should be rewarding. On the other hand prompt treatment of small melanocytic lesions will lead to overtreatment of potentially

benign lesions. In the meantime, that is, as long as these studies have not been performed, it is quite certain that radiotherapy will often be applied in selected cases in which vision can be preserved and total tumor control can be established, as radiotherapy with either charged particles or episcleral plaques has become the first choice of treatment of uveal melanoma patients in most of the large ophthalmic oncology centers (see 1.1.2). In addition, as was mentioned in the same paragraph, the relative advantage of mere observation in cases of small primary uveal melanomas, advocated by some authors, appears questionable as long as we are not certain about the long-term survival of patients having such a small melanoma (Shields, 1995).

Turning to the question of the origins of metastases, in Chapter 2 the controversial issue of pre-enucleation irradiation was dealt with (see also 1.3.1). Zimmerman's hypothesis (1978) that the high mortality rate in the second to fourth year following enucleation is the result of micro-metastatic seedings induced by the physical manipulation of the tumor-containing eye during the surgical procedure does probably not hold. As Manschot pointed out, the high mortality peak might also be explained by the natural history of uveal melanomas, in which case this peak will be the result of micro-metastatic seedings before enucleation; he argued, based on doubling-times, that a direct effect of enucleation itself can only be seen after 6 years. Therefore he advocated the pre-enucleation radiotherapy to study this potentially metastatic effect of the surgical procedure itself. Chapter 2 showed that pre-enucleation radiotherapy in the first long-term follow-up study (7.5 years) did in fact not demonstrate a beneficial effect on survival, which makes Manschot's hypothesis on the origins of the micro-metastases more plausible. At any rate, a 10 to 15 year follow-up study should settle this issue at an even more reliable level. As mentioned in Chapter 1 and in continuity with the preceding paragraph, it is interesting from a theoretical point of view that especially cases of small melanomas will (possibly) benefit from pre-enucleation irradiation because these can potentially be cured, in contrast to cases of large tumors, for the latter are more liable to already having given way to micro-metastatic seedings before the initial treatment (Hungerford, 1993). With regard to the survival rate of patients suffering T1 tumors, the study reported in Chapter 2 showed a non-significant difference between the pre-enucleation irradiation and the control group, but this T1 follow-up group was actually too small to answer

this question. For that matter, the follow-up of patients, who have been treated by pre-enucleation radiotherapy will be continued.

On the morphology of uveal melanoma cells, Chapter 3 reported that primary and metastatic uveal melanomas differ significantly in cell type: metastatic lesions consist of more aggressive, undifferentiated tumor cells than the primary tumor they derive from. In clinical practice, this is important mainly with respect to patients having undifferentiated liver metastases with unknown primaries. In such cases the relatively high percentage of HMB-45 staining of uveal melanoma-related metastases can be helpful in differentiating these from other non-melanocytic lineage derived tumors. Apart from this practical side, it is interesting to hypothesize on the differences in cell type between the primary and the metastatic lesions. On the one hand, it can be expected that epithelioid cells are more metastatically potent than spindle cells, because the epithelioid cell type is associated with a higher mortality rate (Luyten, 1995; Gamel, 1993, Coleman, 1993). On the other hand, however, primary uveal melanomas consisting purely of spindle cells are able to metastasize as well. Furthermore, the cells of one metastatic lesion may show a epithelioid-like morphology while cells of another lesion in the same patient may display a spindle cell pattern (see Chapter 3). Whether these morphological changes are related to the genetic instability of the tumor cells and thus with tumor progression or to micro-environmental changes is unknown. More likely is however that these morphological changes are to a certain extent the result of genomic abberations and occur in concordance with their intrinsic metastatic potency and proliferation rate. Whether or not these changes can be explained by malignant transformation of differentiated melanocytes, which will de-differentiate or by malignant transformation of immature stem cells, which will differentiate to a certain extent, is unclear for uveal melanomas.

The fact that an increasing number of uveal melanoma patients is nowadays treated by radiotherapy implies that histopathological tumor material is often not available for analysis. Therefore, prognostic factors, will have to be deduced mainly from clinical observations. In summary of section 1.3.2, investigations that were performed to identify patients at a high risk of developing metastatic disease, carried out in groups of patients treated by enucleation or radiotherapy and in groups of patients with small dormant and non-dormant uveal melanomas, elucidated the

following main clinical prognostic factors: largest tumor diameter (LTD), anterior location and presence of extraocular extension of the tumor, and advanced age at the time of the initial treatment and male sex of the patient. Of patients treated by radiotherapy or other conservative treatment modalities, additional histopathological parameters such as cell type and proliferation markers (MIB1, MYC) could hypothetically be obtained by means of fine-needle-aspiration biopsies (FNAB). However, these parameters could not be used as prognostic factors anyway for these cytological specimens are not representative for the whole tumor.

Another possibility to identify patients at risk as pointed out in 1.3.2, is the yet hypothetical possibility of the early detection of disseminated tumor cells, which foremostly would be a great advantage with respect to the choice of to whether or not assess systemic treatment. To detect these cells, the minimal detection of tyrosinase in peripheral blood by the polymerase-chain-reaction technique might be the solution (Tobal, 1993), despite recent contradictory results (Foss, 1995;). Still it appears that the minimal detection of a panel of circulating melanoma-specific and associated antigens in peripheral blood could possibly prove even more sensitive than that of tyrosinase alone. Apart from this, it would be a considerable gain if ultrasonic techniques could be developed to determine the presence of vascular networks, which has been shown to be a highly significant histopathological prognostic factor (Damms, 1995).

The value of another possible prognostic factor in uveal melanoma, that of *NM23* expression, was investigated in Chapter 5. In this study it appeared that the association between immunohistochemically assessed NM23 expression and survival is only of "borderline significance". The hypothesis that NM23 is related with metastatic behavior in uveal melanoma is supported by another study. Unpublished data of Ma and associates on uveal melanoma cell lines transplanted intracamerally in nude mice report a strong inverse correlation between the level of *NM23* mRNA expression and NM23 antigen expression on the one hand and the development of metastases on the other. Furthermore, the survival time of the nude mice in this study shows to be correlated with the *NM23* gene expression (Ma, 1996; submitted for publication). At any rate, as was further mentioned in Chapter 5, the immunohistochemical assessment of NM23 antigen expression might not altogether be that informative, for it is likely that the *NM23* gene is involved in the metastatic

process at another, transcriptional or post-transcriptional level (Postel, 1994; id 1993). Consequently, in order to determine the precise prognostic value of *NM23*, investigations that focus on this particular level should be undertaken.

In vivo as well as in vitro studies show, turning to another subject of section 1.1.3, that in most cases of metastatic uveal melanoma the liver is involved (Kath, 1993; Niederkorn 1993; Rajpal, 1983;), whereas cutaneous melanomas preferentially metastasize to the lungs and other organs (Einhorn, 1974). This difference in organ-specific metastasizing between the uveal and cutaneous melanoma suggests that site-designation in metastasis is not an at random event. The predilection of certain tumors to metastasize consistently to specific organs must be the result of organ-specific homing and hence, recognition of vascular endothelial receptors unique to those organs by circulating tumor cells (Niederkorn, 1993; Nicolson, 1988). The association between high NCAM expression with both rapidly metastasizing primary uveal melanomas and metastatic lesions themselves, demonstrated in Chapter 4, shows that cell adhesion molecules may play an important role in the homing capacity of disseminating uveal melanomas. Furthermore, lack of HNK-1 expression in the metastatic lesions was in this study proved to be strongly associated with liver involvement, which may be an indication that the NCAM isoforms lacking the HNK-1 epitope is involved in the organ-specific metastatic behavior of uveal melanomas.

We will now turn to the experimental studies presented in the latter half of the thesis. The establishment and characterization, described in Chapter 6, of two primary and three metastatic uveal melanoma cell lines - of which until recently indeed only very few respectively none were available (see 1.3.3.1) - opens the possibility to initiate studies for a better understanding of the fundamental processes regulating the uveal melanoma metastatic phenotype and the development of models that accurately reflect the pathogenesis of malignancy and predict responsiveness of uveal melanoma in humans. Thereby these cell lines might open the door to the development of new therapeutical modalities of metastatic uveal melanoma, which was the third and eventually main aim of this thesis. In fact, apart from studies by the author and associates, these cell lines are now being used by several other research groups conquering various problems on carcinogenesis, tumor progression and tumor immunology.

As was described in Chapter 7 (see also section 1.3.3.2), these cell lines were used to determine the expression of the melanoma-specific and associated antigens *MAGE1, -2 and -3, GP100* and *tyrosinase* in uveal melanoma. Two cell lines that were found to express all five antigens, OCM-1 and OMM-1, could for this reason probably be applied in specific active immunotherapy, analogue to metastatic cutaneous melanoma, which is now experimentally treated by means of immunotherapy consisting of repeated injections of irradiated tumor cells expanded in tissue cultures. These cutaneous melanoma vaccines have proved, at least in murine models, to indeed be able to induce a long-lasting specific anti-tumor immunity (Dranoff, 1993). In order to stimulate systemic anti-tumor activity, tumor cells dedicated for immunotherapy have to be transfected with either IL-2 or GM-CSF (Dranoff, 1993). Because the whole procedure of culturing biopsied melanoma cells and carrying out the cytokine gene transfection and irradiation is rather time consuming, some investigators of cutaneous melanoma immunotherapy make use of allogenic instead of autologous cell lines (Stevens, 1995; Mitchell, 1994). Accordingly, as the survival time of uveal melanoma patients with metastatic disease is limited, these patients will probably also benefit most from allogeneic tumor vaccines.

However, before the cell lines OCM-1 and OMM-1 can be used in practice for specific immunotherapy, several additional studies will have to be carried out, especially on the susceptibility of these cell lines to the various vectors of the immune system and vice versa, on the reactivity of these vectors to the cell lines, for instance in animal models (see sections 1.3.3.2-3). One such study was presented in Chapter 8, which is also the second example of the use of our cell lines. In this study, the cell lines were transplanted intracamerally into nude mice, and thus it was showed that the down-regulation of MHC class I molecules in uveal melanoma cells raises the susceptibility of these cells to natural killer (NK) cells. In other words, when CTL-antitumor response is not induced by lack of an appropriate MHC class I molecule, uveal melanoma cells still induce an immune reaction in the sense that they are recognized and killed by NK's. Whether it might indeed be possible to employ NK-cell activity in the treatment of patients suffering distant uveal melanoma metastases, other than with a general stimulation of the cellular response by for example IL-2, is yet unclear.

In the final Chapter, Chapter 9, the cell lines were used in a novel chicken embryo model in which growth and metastatic process of uveal melanomas can be studied. The basic idea of developing this model is that human uveal melanoma cells can grow in the eye of an immunocompetent animal. The human cells have to be transplanted at an orthotopic or a heterotopic site before the chicken embryo's immune system has developed, that is amply before day 9; in this model, the human cells taken from the uveal melanoma cell lines were transplanted into the eye of the chicken embryos at day 3.5. Although a high success rate of transplanted tumor cells was reached, the eventually attained number of hatched chicken with a growing intraocular tumor was relatively low. Therefore, although this model might be useful to the study of intraocular growth of human uveal melanoma cells, it is probably not reproducible enough to be elaborately engaged in the study of metastatic processes and prevention strategies. Better results were obtained in another study, when the cell lines were transplanted into nude mice in collaboration with Niederkorn an associates (Ma, 1995, id, 1996; submitted for publication).

In conclusion, regarding the therapeutic approach of uveal melanoma, it is essential to recognize those patients with a high risk of developing lethal metastatic disease at the time of presentation with a primary uveal melanoma. With respect to this, at present two topics appear to be of main importance. Firstly, the evaluation of more sensitive prognostic factors, so that the choice of treatment may appropriately be tuned to every individual case. The prognostic factors that are seemingly most worthy of further elaboration are the presence of closed vascular networks, the early detection of disseminated tumor cells and the organ-specific homing capacities of tumor cells. Secondly, evidently the treatment modalities themselves have to be further developed. As was argued above, regarding these, active specific immunotherapy for metastatic disease or as an adjuvant therapy by use of the cell lines OCM-1 and OMM-1 appears to be a promising option. Before this type of immunotherapy might be introduced, apparently more studies are required on uveal melanoma cell lines in relation to the immune system, first of all directed to the question whether cytotoxic T-lymphocytes (CTL) indeed recognize and kill these tumor cells. Such studies on CTL recognition and the ability of CTL's to mediate anti-tumor responses are at present underway. When a specific active immunotherapy will have been developed, its effects on the

survival of patients suffering metastatic disease should be assessed in a clinical trial. If an immunomodulation can indeed be induced and the side-effects of such a therapy are minimal, specific active immunotherapy might in the future be applied as an adjuvant therapy to cases of primary uveal melanoma as well. When the effects of such adjuvant immunotherapy, especially with regard to the down-regulation of MHC class I and II molecules after radiotherapy, will stand the comparison with those of enucleation and radiotherapy, we may have taken a step towards a decrease in uveal melanoma-related mortality.

SUMMARY

Uveal melanoma is the most common primary intraocular malignant tumor in adults with an estimated annual incidence in The Netherlands of about 80 to 100. The introductional Chapter points out that to date studies on this type of cancer mainly focused on diagnosis and treatment of the primary tumor. This has resulted in the very low misdiagnosis rate of primary uveal melanoma of less than 1 % today, owed to the application of diagnosis techniques like ultrasonography, fluorescence angiography and especially indirect ophthalmoscopy. The choice of how to manage primary uveal melanoma is quite controversial, and often but to a limited extent directed by long-term survival chances of the patient. In addition to the initially only available treatment of enucleation of the primary tumor-containing eye, several alternative (conservative) treatment modalities such as photocoagulation, local resection and principally radiotherapy have been developed with the major interest of retaining visual acuity and tumor control. However, despite all efforts to develop new therapies, still at least 50 % of the patients with uveal melanoma eventually dies of distant metastatic disease. Once such metastases have been diagnosed, the life expectancy of the patient is very low (2 - 9 months) as metastatic uveal melanoma, which is mostly located in the liver, is a particularly malignant disease for which practically no rewarding treatment is available yet. Only a few therapies which included chemotherapy have proved to extend the median survival time of the patients with about 3 months. On the whole, survival percentages assessed by Kaplan-Meier curves and the Cox analysis are 75%, 65% and 55% after respectively 5, 10 and 15 years, with a peak of melanoma-related deaths in the second to fourth year following treatment of the primary tumor.

The present thesis investigates some of the key elements involved in the metastatic process of uveal melanoma with the aim of contributing to the development of a therapeutic approach that should decrease the high overall mortality rate of 50 %. The three basic points of interest are the origins of the metastases, the formulation of clinical and histopathological factors involved in the metastatic process, and the treatment (and prevention) of uveal melanoma-related metastatic disease.

Chapter 2, regarding the first point, elaborates on the hypothesis that micro-

metastatic spreading is not induced by the physical manipulation during the enucleation of the primary tumor-containing eye, as Zimmerman suggested (1978), but is due to micrometastasis prior to treatment, as Manschot pointed out (1980). Evidence in favor of Manschot's hypothesis has already been given by several studies on the effects on survival of pre-enucleation radiotherapy. The present first long-term follow-up study (7.5 years) demonstrated no difference in survival between a group of patients treated by two fractions of 4 Gy prior to enucleation and the control group, patients who were treated by enucleation alone. This makes Manschot's hypothesis that micro-metastases originate independent of treatment even more liable. However, to settle this issue another study with a follow-up of 15 years is required.

Previous investigation into the identification of patients at a high risk of developing metastatic disease, our second point of interest, elucidated the following main clinical prognostic factors: largest tumor diameter (LTD), anterior location and presence of extraocular extension of the tumor, and advanced age at the time of initial treatment and male sex of the patient. Notably, histological material is often not available now with the increase in conservative treatment modalities, so that prognostic factors indeed have to be deduced mainly from clinical observation.

In the studies presented in Chapters 3 and 4, cells from primary and metastatic uveal melanomas were compared. A significant difference in morphology was demonstrated: metastatic tumors display a higher percentage of aggressive and undifferentiated cells compared to the corresponding primary tumor. Furthermore, it was found that staining by HMB-45 is the most sensitive immunohistochemical marker for this type of melanoma. Next, the presence of neural cell adhesion molecules in the primary and metastatic uveal melanoma cells was determined. The presence of the adhesion molecule NCAM in both aggressive, metastasizing primary and metastatic lesions and the relative paucity of HNK-1 expression in metastatic lesions of the liver together suggest that the expression of the NCAM isoforms lacking the HNK-1 epitope might be an important factor in the organ-specific, liver-directed homing of uveal melanoma. These data show that detailed expression of adhesion molecules in primary uveal melanomas may have promising prognostic value.

Chapter 5, analogous to research on (mainly) mamma carcinoma, studies the prognostic value of the expression of *NM23*, which has been described as a putative

metastasis suppressor gene. However, in the present study on uveal melanoma the prognostic value of the protein expression in the primary lesion appeared to be only of "borderline significance". Yet, as the function of the *NM23* gene and its products is to date unclear, its major function might be appointed at (post-)transcription level, which then has to be investigated in another type of study.

The latter half of the thesis is dedicated to experimental studies. To date, studies on biological behavior and therapies of uveal melanoma, the third and main aim of this thesis, have been hampered by a paucity of uveal melanoma cell lines. Chapter 6 describes the successful establishment of two primary and three metastatic uveal melanoma cell lines, that were subsequently characterized by light and electromicroscopy, immunohistochemical and cytogenetic analysis and then used in the several studies discussed below.

The study reported in Chapter 7 showed that two of these cell lines (OCM-1 and OMM-1) express the three *MAGE*, the *tyrosinase* and the *GP100* genes. Because they express MHC class I molecules as well, these cell lines might be employed in allogenic tumor vaccination, similar to immunotherapies currently applied to patients suffering metastatic cutaneous melanoma. In cases of metastatic uveal melanoma, considering the limited survival time, the use of autologous material appears less feasible. If such specific active immunotherapies might be developed, these could ultimately be applied as adjuvant therapy in cases of primary uveal melanoma too. However, before such immunotherapies might be introduced in clinical practice, cytotoxic T-lymphocyte (CTL) recognition and anti-tumor response have to be determined.

Chapter 8 describes the transplantation of uveal melanoma cell lines into the animal model of the immunodeficient nude mouse. Following recent data on other models, human uveal melanoma cells were transplanted at the orthotopic site, that is intracamerally, after which the model indeed developed distant liver metastases. This experiment further showed that the down-regulation of major histocompatibility complex (MHC) class I molecules in uveal melanoma cells raises the susceptibility of these cells to natural killer (NK) cells. Thus, when CTL antitumor response is not induced by lack of an appropriate MHC class I molecule, uveal melanoma cells still induce an immune reaction in the sense that they are recognized and killed by NK's.

Chapter 9 reports on the establishment of a novel animal model, the chicken embryo. The uveal melanoma cells were transplanted into the model's eye at day 3.5, hence amply before day 9 when it's immune system develops. The transplants were not repulsed, the embryo's eye development was not disturbed and the chickens even hatched, suffering a human uveal melanoma. In spite of the many possibilities it seemingly provides, this model proved to be less reproducible in larger series and might therefore not be practical for elaborate intervention studies.

Finally, the Discussion in Chapter 10 emphasizes that the management of a primary uveal melanoma should be directed by the fact that this disease is eventually lethal in 50% of the cases. In this regard, a large study on the effects on survival of radiotherapy versus enucleation is currently undertaken (COMS). Furthermore, the evaluation of prognostic factors such as vascular networks, detection of circulating disseminated melanoma cells and neural cell adhesion molecules is of major importance, as well as the development of therapies for uveal melanoma-related metastatic disease. With respect to the latter, especially active immunotherapy using allogenic cell lines, reported in Chapters 6 and 7 above, appears to be a most promising starting-point, while the nude mouse appears to be a useful model for the investigation of actual immune reponses to such therapy.

— SAMENVATTING IN HET NEDERLANDS —

Het oogmelanoom is de meest voorkomende primaire maligne tumor van het oog met een jaarlijkse incidentie van circa 80 à 100 in Nederland. In paragraaf 1.1.1, *Inleiding tot het onderwerp* wordt besproken dat met betrekking tot deze vorm van kanker het stellen van de diagnose en de lokale behandeling van de primaire tumor de afgelopen 30 tot 40 jaar de belangrijkste punten van discussie zijn geweest. Dit heeft geresulteerd in een tegenwoordig zeer laag percentage (<1 %) van fout-positieven, ofwel gevallen die ten onrechte worden gediagnostiseerd als oogmelanoom, te danken aan de ontwikkeling van technieken als ultrasonografie, fluorescentie-angiografie en met name indirecte ophthalmoscopie. Van oudsher werd het oogmelanoom behandeld door middel van een enucleatie, dat wil zeggen het verwijderen van het gehele oog. Met het doel de oogbol en gezichtscherpte te behouden is er gestart met behandelingsmethoden zoals fotocoagulatie, lokale verwijdering van de tumor en vooral radiotherapie, die in tegenstelling tot verwijdering van het gehele oog als conservatieve behandeling verder geduid worden. Desondanks is de levensprognose van patiënten met een oogmelanoom nauwelijks verbeterd. Meer dan 50% van alle patiënten krijgt op termijn metastasen, welke meestal gelokaliseerd zijn in de lever. Deze zeer kwaadaardige metastasen zijn tot op heden praktisch onbehandelbaar, en op het moment dat zij klinisch zijn vastgesteld is de levensprognose van de patiënt zeer kort (2 tot 9 maanden, afhankelijk van de lokatie van de metastasen). Er is geëxperimenteerd met diverse therapieën, waarbij slechts enkele behandelingsmethoden waarin gebruik wordt gemaakt van chemotherapie - al dan niet gecombineerd met een operatie of immuuntherapie - een kleine vermeerdering van de gemiddelde overlevingstijd (3 maanden) lieten zien. Overlevingspercentages van patiënten bij wie een primair oogmelanoom is vastgesteld, berekend aan de hand van Kaplan-Meier curves en Cox analyses, bedragen 5, 10 en 15 jaar na de eerste behandeling respectievelijk 75 %, 65 % en 55 %. De hoogste mortaliteit wordt gevonden in het tweede en derde jaar volgend op behandeling van de primaire tumor.

Deze situatie vormt het uitgangspunt van dit proefschrift, zoals wordt aangegeven in paragraaf 1.2, *Doel en bereik van de these* . De gepresenteerde studies

bestuderen verscheidene facetten van het metastaseringsproces in oogmelanoom met als uiteindelijk doel bij te dragen aan het ontwikkelen van meer succesvolle behandelingsprocedures, die met name gericht zouden moeten zijn op het voorkomen van sterfte aan (metastasen van) oogmelanoom. De vragen die hierbij tot leidraad dienen zijn: waarom en hoe ontstaan metastasen op afstand, welke patiënten hebben een verhoogd risico op het ontwikkelen van metastasen, en hoe zouden deze oogmelanoommetastasen behandeld, en uiteindelijk liefst voorkómen kunnen worden?

Hoofdstuk 2, ingeleid in paragraaf 1.3.1, betreft een studie naar het lange termijn-effect van bestraling vóór enucleatie op overleving. Aanleiding tot dit onderzoek was de hoge incidentie van oogmelanoom-gerelateerde sterfte in het tweede en derde postoperatieve jaar, die wellicht verklaard zou kunnen worden door micrometastasen die vrijkomen in de circulatie als gevolg van de fysieke manipulatie van het oog tijdens de operatie (Zimmerman, 1978). Bestraling van de tumorcellen vóór de ingreep zou dan een groot deel van deze cellen vernietigen en daarmee de kans op metastasering verminderen. Op basis van gegevens van verdubbelingstijden van oogmelanoom is door Manschot en Van Peperzeel (1980) echter gesteld dat pas op zijn vroegst 6 jaar na enucleatie een effect van deze operatie kan worden waargenomen en dat de hoge mortaliteit in de eerste jaren volgend op de operatie dus verklaard moet worden door micrometastasen die optreden vóór en onafhankelijk van de ingreep. De (weinige) voorgaande studies op dit gebied ondersteunden steeds Manschots hypothese; deze werkten echter met een relatief korte follow-up. In de in Hoofdstuk 2 beschreven studie werd iedere patiënt minimaal 7.5 jaar gevolgd. Omdat er ook ditmaal geen effect van voorbestraling kon worden waargenomen, kunnen we voorzichtig concluderen dat de enucleatie procedure op zich zelf slechts weinig of geen effect heeft op de overlevingstijd van de patiënt. Dat betekent tegelijkertijd dat de micrometastasen die het begin van het uitzaaiingsproces markeren, zich waarschijnlijk onafhankelijk van (enige vorm van) behandeling voordoen. In de *Discussie* in Hoofdstuk 10 wordt aangegeven dat vermoedelijk ongeveer 15 jaar follow-up nodig is om werkelijk uitsluitsel te kunnen geven over dit onderwerp, de oorsprong van de metastasen en ingrijpen op dat niveau.

Met betrekking tot het bepalen van klinische en histopathologische prognostische factoren, een tweede kernonderwerp van dit proefschrift, wordt in

paragraaf 1.3.2 voorafgaand onderzoek besproken. Dit onderzoek heeft uitgewezen dat de grootte van de primaire tumor (LTD = grootste tumordiameter) verreweg de belangrijkste parameter is met betrekking tot het risico op het ontwikkelen van metastasen. Onder de klinische prognostische factoren vallen onder andere ook lokatie en extrasclerale doorgroei van de tumor en gevorderde leeftijd en mannelijk geslacht van de patiënt, die alle een verhoogd risico opleveren. Histopathologisch lijkt, naast tumorgrootte, ook celtype van belang. Vanwege het toenemend aandeel van conservatieve behandelingen is histologisch onderzoek van tumormateriaal vaak echter niet mogelijk. Op het moment wordt er gewerkt aan een rt-PCR-techniek die middels het aantonen van tyrosinase circulerende melanoomcellen detecteert. Tenslotte is recent een nieuwe, zeer sensitieve en onafhankelijke prognostische factor gevonden: het vóórkomen van vasculaire netwerken in oogmelanomen, waarvoor echter op dit moment nog wel histologisch weefsel nodig is.

In de in Hoofdstuk 3 en 4 gerapporteerde studies werd van een groep patiënten die zijn overleden als gevolg van metastasen op afstand de primaire tumor vergeleken met de corresponderende metastase(n). Allereerst werd in Hoofdstuk 3 een significant morfologisch verschil geconstateerd: metastasen bevatten een veel hoger percentage agressieve en ongedifferentieerde tumorcellen vergeleken met het primaire oogmelanoom waar zij van afstammen. Een deel van de metastasen bestond zelfs volledig uit ongedifferentieerde cellen die geen kenmerken meer vertoonden van een oogmelanoom. Kortom, de morfologie van tumorcellen verandert in de loop van het metastaseringsproces. Uit dit onderzoek bleek verder dat een immunohistochemische kleuring met HMB-45 het meest sensitief is voor het gemetastaseerde oogmelanoom. De studie besproken in Hoofdstuk 4 toonde vervolgens aan dat in zowel primaire als gemetastaseerde oogmelanomen de neurale cel-adhesiemoleculen NCAM en HNK-1 kunnen voorkomen. Adhesiemoleculen zijn essentieel voor de nesteling van tumorcellen in organen op afstand vanwege hun specifieke herkenning van receptoren in het vaatendotheel ter plaatse, zoals onder andere is gebleken uit onderzoek naar huidmelanoom. Nu bleek een verhoogde expressie van NCAM significant gecorreleerd te zijn met primaire oogmelanomen die vroeg metastaseren en met metastasen zelf, terwijl de expressie van de HNK-1 epitoop in de metastasen van de lever vaak sterk verminderd was ten opzichte van de primaire tumor. Dit suggereert

dat een subvorm het adhesiemolecuul NCAM, die niet het HNK-1 tot expressie brengt, mogelijk verband houdt met de orgaanspecificiteit, de voorkeur voor de lever, van het oogmelanoom. Het oogmelanoom metastaseert immers specifiek naar de lever, in tegenstelling tot bijvoorbeeld het huidmelanoom, dat voornamelijk naar de long metastaseert.

In andere typen tumoren is een gen gevonden waarvan het eiwitprodukt mogelijk een metastase-onderdrukkend effect heeft, het *NM23* (niet-metastaserende kloon 23) gen. Verminderde expressie van dit gen blijkt in met name mammacarcinomen sterk geassocieerd te zijn met een verhoogde kans op metastasen en verminderde overleving. Hoofdstuk 5 beschrijft in analogie met deze gegevens een studie naar de expressie van het *NM23*-gen in primaire oogmelanomen en cellijnen. In dit onderzoek werd de genexpressie bepaald op grond van het immunohistochemisch aantonen van het genprodukt, het NM23-eiwit, waarbij een "borderline" associatie gevonden werd tussen het percentage NM23-negatieve velden en overleving. Zoals in Hoofdstuk 10 ook wordt bediscussieerd, zou de associatie tussen NM23 en metastasering/overleving in werkelijkheid echter nauwer kunnen zijn gezien het feit dat er aanwijzingen bestaan dat het *NM23*-gen op een ander, namelijk transcriptioneel of post-transcriptioneel niveau werkzaam is. Het vaststellen van de werkelijke relatie tussen *NM23* en overleving vereist daarom nader onderzoek op deze niveaus.

Met het oog op het ontwikkelen van een behandeling voor gemetastaseerd oogmelanoom, het derde aandachtsveld van deze these, is het voor alles van belang dat er mogelijkheden worden geschapen voor experimenteel onderzoek. Zoals in 1.3.3 uitvoerig wordt uiteengezet, kan het metastaseringsproces immers pas werkelijk uitgebreid bestudeerd worden onder laboratorium-omstandigheden. Tot op heden werd dit echter belemmerd door het feit dat er slechts enkele humane oogmelanoom cellijnen beschreven waren en er daarnaast geen geschikt dierproefmodel beschikbaar was aan de hand waarvan (ingrijpen in) het metastaseringsproces bestudeerd kon worden. De auteur en medeonderzoekers zijn er recentelijk wel in geslaagd twee primaire en drie metastatische oogmelanoomcellijnen te kweken. Hoofdstuk 6 beschrijft deze kweek en de karakterisering van de verkregen cellijnen. De karakterisering werd uitgevoerd door middel van morfologisch onderzoek met licht- en electronenmicroscopie, immunohistochemisch onderzoek en

chromosoomonderzoek door middel van karyotypering. De ontwikkeling van deze cellijnen biedt goede mogelijkheden voor studies naar de biologische eigenschappen van het oogmelanoom; ook kunnen zij gebruikt worden voor studies aan de hand van proefdiermodellen.

In de in Hoofdstuk 7 besproken studie, ingeleid in 1.3.3.2, werd de expressie van enkele melanoom-specifieke en geassocieerde antigenen onderzocht in voornoemde oogmelanoom-cellijnen. Zoals wederom is gerapporteerd met betrekking tot huidmelanoom, spelen deze antigenen, *MAGE1,-2,* en *-3* alsmede *GP100* en *tyrosinase*, een essentiële rol bij de herkenning van melanomen door cytotoxische T lymphocyten, waardoor deze laatste ook in staat zijn de tumorcellen te doden. De genen die coderen voor deze eiwitten komen in het geval van de *MAGE*-genen alleen bij melanomen tot expressie en worden daarom melanoom-specifiek genoemd. In het geval van *GP100* en *tyrosinase* komen zij in alle melanocytaire cellen tot expressie, dus ook in melanocyten, en worden daarom melanoom-geassocieerd genoemd. In de cel wordt na transciptie van deze genen het eiwitprodukt gereduceerd tot een peptide van 8 tot 10 aminozuren, dat in het endoplasmatisch reticulum een complex aangaat met een MHC klasse I molecuul. Dit complex wordt getransporteerd naar de celmembraan, waar het herkend kan worden door cytotoxische T lymphocyten. De huidige studie toont aan dat met name twee cellijnen, OCM-1 en OMM-1, alle antigenen in kwestie tot expressie brengen. Deze twee cellijnen zouden daarom bij uitstek gebruikt kunnen worden voor actieve immunisatie van oogmelanoompatiënten met metastasen op afstand, zoals thans al gebeurt in gevallen van gemetastaseerd huidmelanoom. Overigens is voor oogmelanoompatiënten met metastasen actieve immunisatie met autologe tumorcellen te prefereren boven allogene immunisatie. Gezien de korte levensprognose van de patiënten enerzijds en de voor het kweken, transfecteren en bestralen van de tumorcellen benodigde tijd anderzijds is het praktisch vrijwel onmogelijk om autoloog materiaal te gebruiken. Zoals in Hoofdstuk 10, *Discussie* wordt besproken moet nog vastgesteld worden of cytotoxische T lymphocyten de tumorcellen inderdaad herkennen, alvorens met een klinische proef naar het effect van een dergelijke immuuntherapie gestart kan worden. Studies naar de cytotoxische T lymphocyten herkenning van de oogmelanoom cellen wordt op dit moment nog verder onderzoek verricht. Als actieve immunisatie van

oogmelanoompatiënten met metastasen op afstand inderdaad mogelijk blijkt te zijn en geen ernstige bijwerkingen sorteert, zou deze op den duur ook als ondersteunende therapie naast enucleatie en radiotherapie kunnen worden toegepast in de behandeling van primair oogmelanoom. Voor gevallen van gemetastaseerd huidmelanoom, althans in diermodellen, is beschreven dat een dergelijke therapie een goede anti-tumorimmuniteit oplevert.

Hoofdstuk 8 beschrijft een studie met een proefdiermodel, het naakte muismodel, waarbij oogmelanoomcellijnen in het proefdier werden getransplanteerd. In de naakte muis is de cellulaire lymphocytaire afweer afwezig waardoor allogene en xenogene transplantaten niet worden afgestoten. Uit andere studies is gebleken dat metastasering alleen optreedt indien de tumorcellen worden getransplanteerd naar het orgaan van oorsprong; in dit geval moesten de humane oogmelanoomcellijnen dus getransplanteerd worden naar de voorste oogkamer van de naakte muis. In deze proef werden behalve een geslaagde transplantatie van primaire tumorcellen inderdaad reproduceerbaar metastasen verkregen. De studie toonde bovendien aan dat de gevoeligheid van de getransplanteerde oogmelanoomcellijnen voor "natural killer" cellen groter is in geval van een verminderde expressie van major histocompatibiliteitscomplex (MHC) klasse I moleculen; de proefdieren die getransplanteerd waren met een cellijn die deze klasse MHC verminderd tot expressie brengt ontwikkelden geen metastasen, terwijl deze wel ontstonden bij onderdrukking van de "natural killer" cellen door middel van cyclophosphamide of anti-asialo GM1. Hierbij is natuurlijk van belang dat bij verminderde MHC-expressie ook cytotoxische T lymphocyten de cellen niet herkennen en dus niet doden. In de *Discussie* in Hoofdstuk 10 wordt over dit onderwerp nog opgemerkt dat het vooralsnog onzeker is of NK-activiteit ook in de praktijk ingezet kan worden om metastasen in humane patiënten te bestrijden

In Hoofdstuk 9 wordt een ander proefdiermodel beschreven: een voor de studie van oogmelanoom nieuw model van kippenembryo's. De belangrijkste gedachte achter dit model is dat de transplantatie van de cellen plaatsvindt voordat het immuunsysteem van het kippenembryo zich heeft ontwikkeld, zodat zij als lichaamseigen worden ervaren en zodoende niet worden afgestoten. Het bleek mogelijk om oogmelanoomcellen te transplanteren in het oog van een 3.5 dag oud

kippenembryo, dat wil zeggen ruimschoots voor dag 9 wanneer het immuunsysteem tot ontwikkeling komt. De tumorcellen konden worden opgespoord door immunohistochemische kleuring met HMB-45, NKI/c3 en S100 en bleken aldus inderdaad te groeien in het oog van het kippenembryo. Het bleek bovendien mogelijk het ei te laten bebroeden en uit te laten komen, zodat er kuikens met een humaan oogmelanoom geboren werden. Dit model, dat in principe uitstekende mogelijkheden biedt om groei en afweer van oogmelanomen in vivo te bestuderen, bleek echter niet in die mate reproduceerbaar dat er uitgebreide interventiestudies mee zouden kunnen worden verricht.

Tenslotte wordt, aan het eind van Hoofdstuk 10, *Discussie en toekomstperspectieven,* het belang van het ontwikkelen van nieuwe behandelingsvormen voor oogmelanoom nogmaals onderstreept. Bij constatering van een primair oogmelanoom moet het feit dat dit in 50 % van de gevallen tot de dood leidt, nu nog theoretisch, beslist moeten meewegen in de beslissing omtrent de wijze van lokale en/of toekomstige systeem behandeling. Om beter te kunnen inschatten welke behandeling voor welke patiënt het meest in aanmerking komt is met name onderzoek naar de waarde en het vaststellen van de verschillende prognostische factoren van belang, zoals het zichtbaar maken van de aanwezigheid van vasculaire netwerken in de tumor, het vroegtijdig aantonen van circulerende tumorcellen, en het bepalen van de in deze these besproken spiegels van neurale cel-adhesiemoleculen. Voorts lijken de kweek en de eigenschappen van enkele cellijnen, besproken in Hoofdstuk 6 en 7, veelbelovend met betrekking tot onder andere het ontwikkelen van actieve immuuntherapieën, die ingezet kunnen worden om metastasen van het oogmelanoom te behandelen en wellicht te voorkomen. De gegevens uit en over de verschillende dierproefmodellen kunnen mogelijk van dienst zijn bij de ontwikkeling van een meer succesvolle therapeutische benadering van oogmelanoom.

ABBREVIATIONS

ATP : adenosine triphosphate
CDK : cyclin-dependent kinase
cDNA : copy deoxyribonucleic acid
COMS : collaborative ocular melanoma study
CTL : cytotoxic T-lyphocytes
FAMMM : familiar atypical mole and malignant melanoma
FNAB : fine-needle aspiration biopsy
HLA : human leucocyte agglutinin
HNK-1 : human natural killer -1
IL-2 : interleukin-2
LAK cells : lymphokine activating killer cells
LTD : largest tumor diameter
Mabs : monoclonal antibodies
MHC : major histocompatibility complex
MLN : mean diameter of the ten largest nucleoli
mRNA : messenger ribonucleic acid
NCAM : neural cell adhesion molecule
NDPK : nucleoside diphosphate kinase
NK cells : natural killer cells
NM23 : nonmetastatic clone #23
PB : Phosphate-buffered saline
PCR : polymerase chain reaction
PBL : peripheral blood lymphocytes
PuF : purine binding factor
SCID : severe combined immunodeficient
SSCP : single-strand-conformation polymorphism
TIL : tumor-infiltrating lymphocytes

CURRICULUM VITAE

Op 9 september 1960 werd de schrijver van dit proefschrift geboren te Etten-Leur en behaalde in 1979 zijn eindexamen VWO aan de Katholieke Scholengemeenschap te Etten-Leur. Van 1979 tot 1986 studeerde hij geneeskunde aan de Erasmus Universiteit te Rotterdam. Gedurende zijn studie was hij werkzaam als student-assistent op de afdeling celbiologie waar hij werkte aan een single cell detectie techniek voor de ziekte van Fabry. In 1985 was hij werkzaam op de afdeling Ophthalmogenetica van het Interuniversitair Oogheelkundig Instituut te Amsterdam waar hij in samenwerking met de afdeling Genetica, Erasmus Universiteit Rotterdam een studie verrichtte naar de verschillende uitingsvormen van de ziekte van von Hippel-Lindau. In dezelfde tijd startte hij het vervolgonderzoek naar het effect van voorbestraling bij oogmelanoom patiënten in het Oogziekenhuis Rotterdam. Hierna heeft hij zijn militaire dienst vervuld van 1986 tot 1988 op de afdeling Dermatologie van het Militair Hospitaal A. Mathijsen. Van 1988 tot 1992 was hij in dienst van het Oogziekenhuis Rotterdam als arts-assistent in opleiding tot oogarts. In deze tijd werd het vervolg onderzoek naar de oogmelanoom patiënten voortgezet en maakte hij een start met het kweken van oogmelanoom cellijnen. De laatste 2 maanden van zijn opleiding voltooide hij op de afdeling Oogheelkunde van het Academische Ziekenhuis Rotterdam, waar hij daarna als oogarts werd aangesteld en tot op heden werkzaam is.

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