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Melan-A: A New Immunomarker for Uveal Melanoma

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ABSTRACT

Melanoma-associated antigen (MAA) MART-1/Melan-A has been described in human cutaneous melanoma. The first objective of this study was to investigate the expressivity of MART-1/Melan-A in primary uveal melanomas. 56 formalin-fixed, paraffin-embedded human uveal melanomas were analyzed by immunohistochemistry. MART-1/Melan-A was found to be positive in 73% of cases, with almost 50% of tumours showing a diffuse involvement. The second objective was to investigate the expression of MAA Mart-1/Melan-A and gp100 in primary and metastatic human uveal melanoma lesions developed in albino rabbits. MART-1/Melan-A and gp100 were expressed in all primary lesions, with mainly focal distribution. Conversely, MART-1/Melan-A and gp100 exhibited a homogeneous pattern in the metastatic lesions, which implies upregulation of these antigens during disease progression. It may be postulated that MART-1/Melan-A and gp100 would be effective immunomarkers for immunotherapeutic strategies in human uveal melanoma.

L'antigène associé au mélanome (MAA) Mart-1/Melan-A a été décrit dans le mélanome cutané humain. L'objectif premier de cette étude visait à enquêter sur l'expressivité du MART-1/Melan A dans les mélanomes uvéaux primaires. 56 mélanomes uvéaux humains fixés dans le formol et encastrés dans la paraffine ont été analysés par immunohistochimie. On a découvert que le MART-1/Melan-A a été positif dans 73% des cas, avec presque 50% des tumeurs démontrant une expression diffuse. Le deuxième objectif fut d'enquêter sur l'expression des AAM Mart-1/Melan-A et gp100 dans les lésions de mélanomes uvéaux humains primaires et métastatiques développés chez les lapins albinos. MART-1/Melan-A et gp100 ont été présents dans toutes les lésions primaires avec une distribution principalement focale. Réciproquement, dans les lésions métastatiques, MART-1/Melan-A et gp100 démontraient une distribution homogène, ce qui implique une augmentation de ces antigènes pendant la progression de la maladie. On peut considérer que MART-1/Melan-A et gp100 seraient des immunomarqueurs efficaces dans l'élaboration de stratégies immunothérapeutiques du mélanome uvéal humain.

"Learning is not attained by chance.
it must be sought for with ardour
and attended to with diligence."

- Abigail Adams

"You gain strength, courage, and confidence by every experience in which you really stop to look fear in the face...You must do the thing you think you cannot do..."

- Anna Eleanor Roosevelt

"Our works do not ennoble us:
but we must ennoble our works."

- Meister Eckhart

When I first entered the Pathology Department, two years ago, I had no idea, how many people I would encounter who would affect my philosophy on life, expand my knowledge, and add wisdom to it all. One of the most important lessons I have learned is that you cannot work alone in this world. It is through the efforts of many that one is able to succeed. This project is a symbol of this lesson, for it would not have been possible without the contribution of countless individuals, and I am grateful to have had the opportunity to work with every one of them.

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A wise man once told me that what truly counts is making a contribution to this world, regardless of the size. This project represents a small chapter within an ever-expanding novel. There is still so much work to do, so many roads to travel, but we hope we have added a few footprints in the sand.

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INTRODUCTION

JUSTIFICATION

Uveal melanoma is the most common primary intraocular tumour among adults, affecting approximately 7 people per million per year[1]. It is second in frequency only to retinoblastoma which is predominantly a childhood tumour[2]. However, if we examine the evolution of our understanding of retinoblastoma, it is evident that research and education has allowed the management of retinoblastoma to improve from being an almost uniformly fatal disease to one in which 95% of patients are cured[3].

The same is not true for uveal melanoma, as patient survival from uveal melanoma has not significantly improved over the last four decades[4]. There is a 70% survival rate after 5 years and a 50% survival rate after ten years[5]. This is mainly due to the fact that metastasis from uveal melanoma frequently occurs many years after removal of the primary tumour with the first evidence of metastasis being discovered, on average, at an interval of 6.5 years[6]. Furthermore, there is no metastatic staging for uveal melanoma since this tumour metastasizes hematogeneously, and lymph node involvement occurs secondary to hepatic metastases. Thus a method of recognizing those patients who have micrometastases but who do not have clinical metastatic disease would be of great therapeutic benefit.

In 1978 Zimmerman and co-workers[7] challenged the effectiveness of enucleation in preventing metastatic disease and hypothesized that enucleation may promote metastasis. This prompted the search for better diagnostic techniques and less invasive forms of treatment. As a result, it is now possible to diagnose patients

early and more adequately through fundoscopy, ultrasound and CT scans. Also, the advent of radiation therapy has provided hope to prevent enucleation.

However, progress to free the patient of this tumour remains unchanged and the questions remain. Why does local control of the tumour not prevent metastasis? Why does metastasis occur so late after the removal of the primary tumour? How is it possible for uveal melanoma cells to remain in the body without causing harm for so many years? What causes them to one day begin growing and invading? What are the mechanisms involved? In an attempt to answer these questions, research has begun to focus more importantly on the patient's immunological response to the tumour rather than tumour biology itself. The goal is to understand how a patient's immune system responds to the disease and then to devise immunotherapeutic strategies against the tumour cells. In order to accomplish this understanding, it is necessary to study animal models of uveal melanoma. The albino rabbit implanted with human uveal melanoma cells is the most effective model, at present, since it produces high numbers of animals with metastatic disease[8].

The premise of immunotherapy is to elicit in vivo cytotoxic T lymphocyte-mediated immune responses against specific tumour targets[9]. This treatment can be directed to specific cells without killing normal tissue and without the hazards posed by radiation and chemotherapy. Recent studies in cutaneous melanoma have focussed on detecting melanoma-associated antigens on malignant cells in primary and metastatic lesions[10-14] as well as in the peripheral circulation[15-19]. These studies

have proven to be effective in detecting melanoma-associated antigens, such as tyrosinase, gp100, and MART-1/Melan-A.

Other studies[9,20-23] are now focussing on immunotherapeutic treatment targeted to cutaneous melanoma cells by means of the antigens presented on them. However, in order to achieve success with immunotherapeutic strategies, the antigens chosen as targets for immunotherapy must be adequately expressed on the malignant cells because absence or low expression of these proteins can lead to a poor immune response[10,24].

Although melanoma antigens have been found to be expressed on cutaneous lesions, we cannot simply generalize that uveal melanoma cells will carry the same antigens. It is important to understand the expression of melanoma antigens in uveal melanoma in order to assess the efficacy of immunotherapeutic treatment against these lesions. As important as it is for cutaneous melanoma, the ability to target melanoma antigens by immunotherapy for uveal melanoma is even more important due to the lack of metastatic staging in this tumour.

HYPOTHESIS

In lieu of the previous concepts, the following hypothesis is formed:

It is hypothesized that both Melan-A and gp100 melanoma-specific antigens are expressed by malignant cells of both primary and metastatic uveal melanoma and during hematogeneous dissemination.

These antigens will therefore be effective tumour markers for molecular biology studies involving the detection of circulating malignant cells and subsequently, as cell targets for immunotherapy.

OBJECTIVES

To confirm this hypothesis, the following objectives were established:

Human Studies

1. Monoclonal antibody (mAb) Melan-A expressivity is investigated in uveal melanoma to determine the presence of Melan-A antigen.

2. mAb Melan-A expressivity is compared with mAbs HMB-45 and S-100 protein in order to determine whether mAb Melan-A should be added to the immunohistochemical profile of uveal melanoma.

3. mAb Melan-A is evaluated as a prognostic indicator for uveal melanoma, through correlation with cell type.

Animal Studies

1. Using the albino rabbit uveal melanoma model, the expressivity of both Melan-A and HMB-45 monoclonal antibodies is investigated in primary and metastatic uveal melanoma.

2. The heterogeneity of Melan-A and gp100 antigens is determined in metastatic uveal melanoma as compared to the primary tumour to assess their potential as targets for immunotherapy.

LITERATURE REVIEW

UVEAL MELANOMA

As previously mentioned, uveal melanoma is a rare tumour, with an incidence of 6 - 7 cases per million people per year[1]. However, it remains the most common primary intraocular malignancy seen in adults. The tumour arises from melanocytes located in the iris, the choroid, and the ciliary body, which are the structures of the uveal tract. It is necessary to emphasize that the eye contains two types of melanocytic cells; the aforementioned uveal melanocytes as well as the pigment epithelial cells of the retina, ciliary body, and iris. Nevi and malignant melanomas arise from the uveal melanocytes, whereas tumours of the pigment epithelium are referred to as adenomas and carcinomas[4].

Risk Factors

The etiology of uveal melanoma remains to be elucidated, however, there are several risk factors associated with the disease. These include age, race, gender, geographic factors, genetics, and predisposing lesions.

Age. A compilation of the cases on file at the Registry of Ophthalmic Pathology at the Armed Forces Institute of Pathology (AFIP), determined that the incidence of uveal melanoma increases as one ages, with the median age at presentation being 53 years[4]. Uveal melanoma is rarely seen in young patients. Shields[25] reports that less than 1% of patients present under the age of 20 years.

Race. Race is an important risk factor since there is an inverse relationship between racial pigmentation and incidence of uveal melanoma. Uveal melanoma is more frequently seen in Caucasians, particularly blue-eyed, blonde individuals. The incidence for this population is approximately 8.5 times greater than in blacks[26]. Margo and McLean[27] observed that only 1% of African Americans presents with this tumour while African natives have an even lower incidence. The incidence among the Asian population was found to lie between that of African Americans and Caucasians.

Gender. Uveal melanoma more commonly affects men than women. A review of 4,995 cases at the AFIP Registry of Pathology determined that male patients made up 55% of all uveal melanoma cases while 45% were female[4]. Epidemiological studies performed in Canada[28] and the United States[3] support this finding. The reason for the higher incidence rate in men is not known.

Geographic Variations. The geographic variations in the incidence of uveal melanoma around the world reflect the different racial groups in certain areas rather than actinic exposure, such that, there is a higher incidence in Scandinavian countries as opposed to Africa[4,29]. Thus, sunlight exposure is not considered a major risk factor in the etiology of uveal melanoma. This finding is supported by the fact that most of the ultraviolet light arriving at the eye is absorbed before it even reaches the uveal melanocytes.

Genetics. The genetics of uveal melanoma are not well defined, as the majority of primary uveal melanoma cases are sporadic. However, in rare cases, uveal melanoma may be familial or associated with a predisposing lesion[30]. Familial uveal melanoma is believed to be inherited in an autosomal dominant mode, however it is rare for many individuals in a given family, over different generations, to be involved[30]. In fact, familial uveal melanoma comprises only 0.6% of all uveal melanoma patients[31].

Cytogenetic changes in uveal melanoma are characterized by several chromosome abnormalities, such as trisomy 8 and monosomy 3 and 6[32]. This may suggest a deletion of a tumour suppressor gene on chromosomes 3 and 6, while multiplication of an oncogene may be implicated on chromosome 8.

Molecular genetic studies have shown an absence of mutations in the N-ras proto-oncogene[33]. Also, p53 mutations have been implicated but they are not the same mutations as found in cutaneous melanoma[34]. Cystine to Thymidine conversions, which are characteristic of ultraviolet light damage, and are frequently seen in cutaneous melanoma, are not present in uveal melanoma. These two findings suggest that ultraviolet light may not play a role in the pathogenesis of uveal melanoma.

Predisposing Lesions. Yanoff and co-workers[35] discovered that choroidal nevi could predispose to uveal melanoma. Although these nevi are composed of atypical

melanocytes, they do possess a benign nature. Based on the high prevalence of uveal nevi and the low incidence of uveal melanoma, it can be estimated that the rate of transformation of nevi to melanoma is approximately, 1 per 10,000 to 15,000 per year[29].

The presence of congenital oculodermal melanocytosis (Nevus of Ota) and ocular melanocytosis may also increase one's risk of developing uveal melanoma[36]. This former lesion is caused by an increase in pigmented melanocytes in the deep dermal tissues of the eyelids, while the latter is due to an excess accumulation of melanocytes in the uveal tract, as well as in the sclera and episclera. Congenital oculodermal melanocytosis is more prominent in Blacks and Asians than Whites[29]. However, ocular melanocytosis is seen in people of all races, but the transformation of this lesion to uveal melanoma occurs more readily in the white population.

Clinical Presentation

There are four clinical stages of uveal melanoma[4,29]. These stages correlate with size of the tumour as well as the histopathological features of the tumour. Stages I & II represent the early stages of the disease and correspond to small tumours. These stages consist of asymptomatic tumours as well as cases with symptoms relating to loss of vision. As the disease progresses (stage III), ocular symptoms such as an elevation in intraocular pressure, inflammation, and pain are observed. Stage IV includes symptoms of extraocular extension, such as proptosis, and occasionally

the presence of a subconjunctival mass. These later stages are associated with larger tumours.

Tumour size is classified according to the largest tumour dimension (LTD). Small tumours have a discoid shape (Figure 1) and are less than 10mm in the LTD. Medium tumours range from 11 - 15 mm, while any mass over 15mm is categorized as a large tumour. Both medium and large sized tumours tend to have either a collar button (Figure 2) or mushroom-shaped appearance (Figure 3). An exception to this usual growth pattern is the diffuse melanoma, which extends itself around the uveal tract without protruding into the centre.

Histopathology

Callender's[37] histopathological study of 111 cases of choroidal and ciliary body melanomas was the first to describe the different cell populations in uveal melanoma. McLean and co-workers[38,39] later modified this classification to the one presently used, known as Callender's Modification. This is the universally accepted classification for Ophthalmic Pathologists. It distinguishes between benign uveal melanocytic lesions, termed nevi, and malignant uveal melanocytic lesions. Furthermore, it classifies uveal melanoma into two main cell types; spindle and epithelioid. However, malignant melanocytes exist within a spectrum ranging between these two extremes and tumours containing both categories of cell types are described as mixed cell tumours. In fact, 48% of tumours are diagnosed as mixed cell type[40].

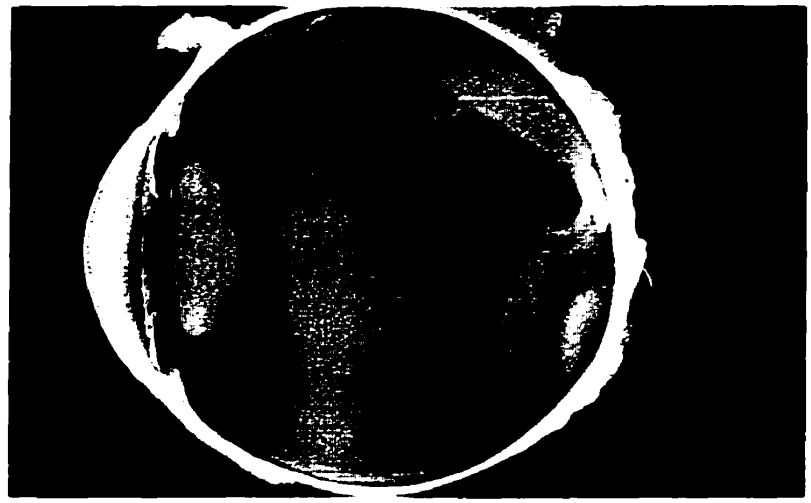

Figure 1. Small, discoid-shaped choroidal melanoma (1108X).

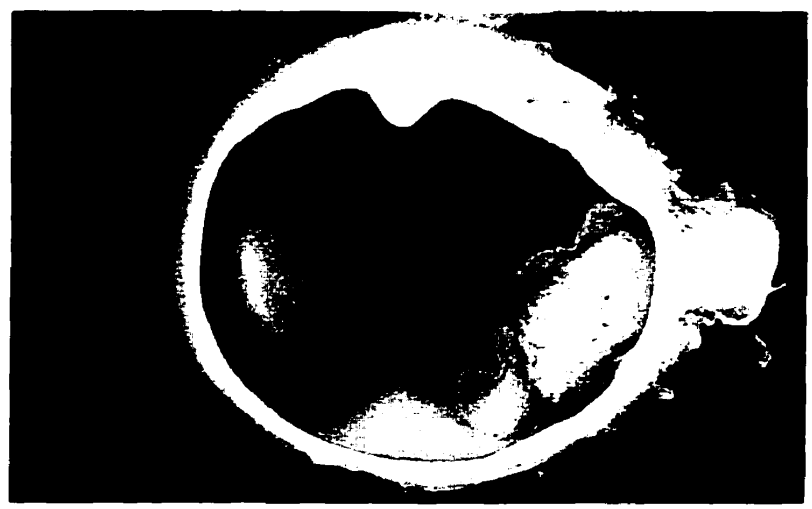

Figure 2. Medium, button-shaped choroidal melanoma with overlying retinal detachment (1108X).

Figure 3. Large, heavily pigmented, mushroom-shaped choroidal and ciliary body melanoma with overlying retinal detachment (1108X).

Spindle cells are highly cohesive, fusiform cells with small nuclei which may or may not contain nucleoli (Figure 4). They often give the appearance of forming a syncytium, since their cellular borders, as defined by the plasma membrane, cannot be distinguished. Small tumours, seen in the early stages of disease are composed of these well-differentiated melanocytes.

Epithelioid cells are large, polyhedral cells with abundant cytoplasm, and containing large nuclei with large, round nucleoli (Figure 5). They are relatively pleomorphic as compared to their spindle cell counterparts. Also, epithelioid cells are spread out in the extracellular matrix architecture, demonstrating a lack of cohesion, and allowing identification of their distinctive cell borders. Occasionally, multinucleated epithelioid cells may be observed. These poorly differentiated melanocytes are mainly seen in larger tumours, reflecting a more aggressive nature of the tumour.

Immunohistochemical Profile

Routinely, uveal melanoma is easily identified through light microscopy. However, on occasion it may be difficult to differentiate melanoma from metastatic carcinomas, schwannomas, and neurofibromas[41]. Immunohistochemical techniques are very important in these instances in order to make a definitive diagnosis of melanoma. To date, the immunohistochemical profile of uveal melanoma consists of HMB-45, S-100 protein, and Neuron-specific enolase (NSE). In a comparative study of these three immunomarkers, Burnier and co-workers[42] found HMB-45 to be the

Figure 4. Uveal melanoma, spindle cell type: highly cohesive, fusiform cells with small nuclei (H&E, 700X).

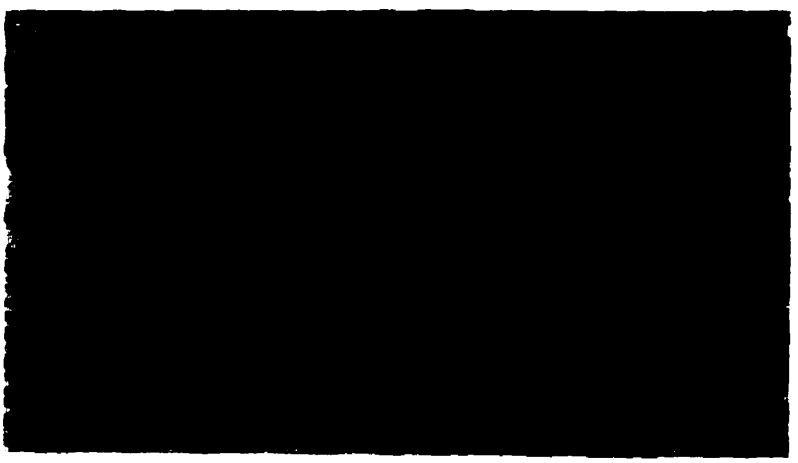

Figure 5. Uveal melanoma, epithelioid cell type: highly pleomorphic cells with large nuclei (H&E, 1120X).

most specific marker for melanocytic lesions of the uveal tract, including nevus and melanoma. Antibodies to the Melan-A antigen have been extensively investigated in cutaneous melanoma lesions[12,13,43,44,45]. However, studies investigating Melan-A antibodies in uveal melanoma are limited. De Vries and co-workers[46] as well as Nicotra and co-workers[44] investigated Melan-A expression in cryostat sections of uveal melanoma with conflicting results. To the best of our knowledge, the expression of Melan-A in a large series of formalin-fixed, paraffin-embedded uveal melanomas has not yet been evaluated. The currently important immunomarkers for uveal melanoma are outlined below.

HMB-45. Adema and co-workers[47,48] discovered that HMB-45 recognizes a glycoprotein of 100 kDa known as gp100. However, another study[49] suggests that HMB-45 recognizes a 3-35kDa melanosome-associated sialated glycoprotein. HMB-45 specificity for melanocytes is demonstrated by immunoelectron microscopy studies[50-53] which reveal that HMB-45 reactivity is restricted to melanosomes of malignant melanocytes. It was observed that HMB-45 stained early stage (I, II, III) melanosomes more intensely than late stage (IV) melanosomes. These studies demonstrate that HMB-45 specifically stains melanosomes as opposed to diffuse cytoplasmic antigen, as described by light microscopic immunohistochemical analysis. Tattjes and co-workers[50] discovered that pretreatment of sections with neuraminidase caused immunolabelling with HMB-45 to be drastically reduced or, in some cases, absent. This supports the idea[50] that HMB-45 is partially composed of sialic acid and that sialylation of the antigen is crucial to HMB-45 binding.

Cheng and co-workers[54] suggested that HMB-45 expression is inducible and closely related to the functional activity of melanocytes. This was determined after irradiated epidermal melanocytes stained positively for HMB-45. Irradiation may lead to morphological changes of the cell, which may imply cell activation. Cell activation in turn may lead to melanosome production. In earlier studies, Skelton and co-workers[55] correlated HMB-45 staining with melanosome production.

Disconcerting is the fact that several studies[56,57] found renal angiomyolipomas (RAML) and smooth muscle cell tumours[58] to be immunoreactive for HMB-45. Zimmer and co-workers[59] also found HMB-45 to be positive in gliosarcomas, primitive neuroectodermal tumours, ependymoma, malignant schwannomas, and intracranial hamartomas, while Hancock and co-workers[60] found HMB-45 reactivity in some adenocarcinomas. However, when confined to diagnosing tumours of the eye, non-melanocytic ocular tumours revealed no HMB-45 staining[42,51]. Therefore, in distinguishing between melanocytic and non-melanocytic ocular tumours, HMB-45 is an excellent immunomarker with greater than 95% positivity[42].

S-100 Protein. This antibody identifies an acidic protein isolated from cow brain that is composed of two subunits; alpha and beta[61]. The alpha unit reacts with axons and melanocytes, whereas the beta subunit is found in schwann cells[62]. S-100 protein is sensitive to both benign and malignant cutaneous and ocular melanomas[12,42,63]. However, S-100 Protein does not stain these tumours with the

same specificity as HMB-45[42,63,64]. S-100 protein is not a melanoma-specific marker, therefore positive immunostaining cannot be considered as unequivocally diagnostic of melanoma. Neural tumours, certain types of histiocytic derived neoplasms, and a variety of carcinomas and sarcomas are positive for S-100 protein[42,64].

Melan-A. Monoclonal antibody Melan-A recognizes the melanoma-associated antigen MART-1/Melan-A which is a melanosomal protein[13]. MART-1/Melan-A was shown to stain primary cutaneous melanoma lesions and pigmented nevi in an homogeneous fashion[12,13,44]. In contrast, metastatic cutaneous lesions stained heterogeneously[12,13,45]. This may suggest that MART-1/Melan-A is downregulated during melanoma progression. Its specificity in cutaneous melanoma is comparable to S-100 protein, and more reliable than HMB-45[12].

Prognostic Factors

Several clinical and histopathological observations aid in determining the prognosis of uveal melanoma. These include tumour size and location, cell type, size of nuclei and nucleoli, lymphocytic infiltration, mitotic figures, architecture of the microcirculation, and extrascleral extension[39,65-67].

Clinical

The most important clinical prognostic factors are tumour size and location, since they inform about tumour aggressivity and provide a good basis upon which to predict possible metastatic spread.

Tumour Size. Tumour height was initially considered the best indicator of tumour size, however, McLean and co-workers[66] concluded from a study of 3,432 cases that the maximum measurement in any one dimension correlated better with prognosis. This explains the poor outcome associated with diffuse melanomas. In a study of 4,410 uveal melanoma cases McLean[67] found that the 20 year survival rate was approximately 78% for small tumours, 55% for medium tumours, and 30% for large tumours. A recent study also showed that small choroidal melanomas with certain risk factors have a greater potential for metastasis than previously realized[68].

Tumour Location. Uveal melanoma arises more frequently in the choroid and is less commonly seen in the iris and ciliary body. Iris tumours do have a better prognosis since they are visible and can be detected early. On the other hand, tumours of the ciliary body carry a worse prognosis due to the highly vascularized nature of the structure.

Extraocular Extension. The sclera provides a tough barrier against the local invasion of uveal melanoma. Therefore, aggressive tumours tend to invade through

the vortex veins as well as the ciliary arteries and nerves. Uveal melanoma very rarely extend through the optic nerve[4,29]. Extraocular extension is observed in large tumours generally composed of epithelioid cells and an intricate microcirculation. Therefore, patients presenting with this characteristic tend to have a poorer prognosis.

Genetics. White and co-authors[69] report that the simultaneous presence of chromosome 3 and 8 abnormalities is associated with a poorer prognosis. However, when each appeared separately, the risk of a poor outcome was the same as if neither abnormality were present. In quite a surprising manner they discovered that an abnormality on chromosome 6 has a protective effect, as patients with the abnormality faired better than those without it. Furthermore, this and other reports[70,71] found that abnormalities in chromosomes 3 and 8 usually occur together and are associated with ciliary body tumours, whereas abnormalities in chromosome 6 occur alone and are associated with choroidal tumours. These reports may support evidence linking tumour location and genetics for prognosis of uveal melanoma.

Other clinical prognostic parameters include tumour margin location anterior to the equator of the eye, older age, male gender, and tumour-induced glaucoma, which are associated with a poorer life prognosis[65].

Histopathological

Cell Type. Cell type correlates with tumour size and is the single most important prognostic factor in uveal melanoma. Smaller tumours composed of spindle cells

offer a better prognosis for the patient, resulting in a 22% death rate due to metastasis while larger, epithelioid cell tumours carry a worst prognosis with the death rate due to metastasis increasing to 62%[40].

Mean Diameter of the Ten Largest Nucleoli (MLN). MLN cannot be easily assessed since it requires the use of image analysis equipment. This factor causes the method to be sensitive and explains the discrepancy between different investigators. Several studies[72,73] found an increase in MLN to be associated with a poorer prognosis, while others[74,75] found MLN to be an insignificant prognostic indicator.

Mitotic Figures. Mitotic figures are rare in uveal melanomas, however they are more frequently seen in epithelioid cell tumours than in spindle cell tumours. McLean and co-workers[76] found that it is best to observe mitotic figures under magnification of 40 high-power fields. Several investigators have found mitotic figures to be of prognostic significance since they are indicative of cellular proliferation[76-79].

Microcirculation. Folberg and co-workers[80] demonstrated that uveal melanoma tumours may be composed of a variety of microcirculation patterns. They main contain straight vessels, parallel straight vessels, cross-linking parallel vessels, vascular arcs, arcs with branching, loops, and networks. Fibrovascular loops are branches of blood vessels that completely surround lobules of tumour, thereby

forming loops. When loops are observed in groups of two or more, these are termed networks.

In a preliminary study of 40 patients Folberg and co-workers[78] showed that the presence of vascular loops was associated with death due to metastasis. A larger study[79] of 234 patients subsequently showed that the presence of loops resulted in a 55.4% survival rate after ten years, whereas tumours lacking these structures demonstrated a patient survival rate of 91.1%. Microcirculation networks were the most significant factor when determining prognosis. The presence of networks correlated with a 50.7% survival rate over ten years. Another study[72] comprising 496 cases confirmed the observations of Folberg and co-workers. Therefore, the microcirculation architecture can be used as an indicator of tumour progression. The more advanced the architecture, the worst the prognosis.

Lymphocytic Infiltration. Tumour infiltrating lymphocytes (TILs) in uveal melanoma are predominantly T-cytotoxic/suppressor cells[81]. De la Cruz and co-workers[77] evaluated the prognostic significance of these TILs. They discovered that the presence of infiltrating lymphocytes is seen in 14 – 20% of uveal melanoma cases, and was associated with death due to metastasis[77]. Whelchel and co-workers[82] also concluded that there was a positive correlation between T-lymphocytic infiltration and death due to metastasis. This is the opposite finding to cutaneous melanoma. However, the discrepancy should not be so unexpected when the anatomy of the two lesions is considered along with the knowledge of their modes of

dissemination. Metastasis from cutaneous melanoma initially spreads via the lymphatic system, however, there is no lymphatic drainage in the eye or the orbit and metastatic spread from uveal melanoma is hematogenous. Since the eye is considered an immune-privileged site, it was suggested that in order to mount a T-cell mediated immune response, malignant cells must first disseminate into the blood[77,82]. These viable circulating tumour cells would be the antigen source needed for immune activation[77,82]. This evidence accounts for the fact that TILs carry a worst prognosis for patients with uveal melanoma. It should be emphasized however that once lymphocytes are present within the tumour, they are believed to have a beneficial effect[83].

Metastasis, Treatment, and Survival

The treatment of choice for uveal melanoma depends upon the size of the tumour. Small tumours tend to be followed and growth is monitored. If no growth is seen, no treatment is undertaken. If these small tumours begin to enlarge, then radiation is applied in order to kill the neoplastic cells while still preserving vision and aesthetics for the patient. Unfortunately, if the tumour continues growing, then enucleation is performed. This is also the case for patients who have large tumours at first presentation. Systemic work-up consisting of several liver function examinations are performed each year to assure no metastatic disease. In a study of 2,627 cases from the Registry of Ophthalmic Pathology (AFIP), Zimmerman and co-workers[84] discovered that the proportion of patients being diagnosed earlier on has increased, while those diagnosed at later stages of disease has decreased. Although

this is a promising sign, it was also concluded that despite earlier treatment, there was no significant improvement in survival[84,85].

As discussed earlier, metastasis from cutaneous melanoma initially spreads via the lymphatic system, however, there is no lymphatic drainage in the eye or the orbit. Therefore, uveal melanoma cells metastasize hematogeneously and preferentially to the liver. Also, unlike cutaneous melanoma which metastasizes to regional lymph nodes and systemic involvement can be traced, there is no such staging in uveal melanoma. Ophthalmologists rely upon prognostic factors to identify patients who are at greatest risk of developing metastatic disease. As of yet, there is no prevention.

Metastasis from uveal melanoma can occur anytime up to 20 years after treatment. It is unlikely that the tumour will metastasize after this time. The first evidence of metastasis is discovered, on average, 6.5 years after removal of the primary tumour[6]. Furthermore, only 1% of uveal melanoma patients show evidence of metastasis at presentation, yet 40 - 50% of patients will develop hepatic metastasis[7,76,85,86]. The five, ten, and fifteen year survival rates for uveal melanoma are approximately 72%, 59%, and 53% respectively[5,87]. It is possible that many patients already have subclinical hepatic metastases that were not detectable upon routine systemic examination[85]. Once a metastatic site is detected clinically, the life expectancy is between two to seven months and fatal in all cases[88]. Surgical resection

of the affected hepatic site coupled with chemotherapy is the best method of improving survival[86,89].

The Collaborative Ocular Melanoma Study (COMS)[90] reports that there is no survival difference between patients who underwent pre-enucleation radiation (74%) or enucleation alone (72%). Gunlap and co-workers[91] also conclude that pre-enucleation irradiation does not improve survival in patients with uveal melanoma. This data suggests that micrometastases occurred before treatment. This also weakens the argument[7] that enucleation causes metastatic dissemination since the patients who underwent radiation before enucleation had the same survival rate.

UVEAL MELANOMA ANIMAL MODELS

The need for animal models to study uveal melanoma is very important due to the low incidence of uveal melanoma[92]. It is impossible to study this tumour unless the eye is enucleated. Also, these tumours are not representative of all uveal melanomas since tumours obtained at enucleation are usually quite large and depict late stage disease. It is also necessary and important to study animal models of uveal melanoma in order to better understand the tumour-host interaction as well as the biology of metastatic dissemination.

In order to design a model which closely relates to uveal melanoma in humans, research has concentrated in developing inducible models. Studies have focussed on developing uveal melanoma by means of viruses[93] and chemicals[94].

However, these models were only sufficient to study the early stages of disease. A transgenic mouse model[95] was also inadequate, since although a tumour of epithelioid cells was developed, it originated from the retinal pigment epithelium.

Other inducible models were developed that were based on implanting tumours. Greene and co-workers[96], who took a pigmented cutaneous melanoma found in hamsters and implanted it into the eye of a rabbit, first developed this model, known as "Green Melanoma." This model allowed for the development of uveal melanoma as well as distant metastases. Over the years, this model has been modified to the one developed by Kan-Mitchell and co-workers[97] who implanted human uveal melanoma cells into the eyes of rabbits immunosuppressed with cyclosporin A. Several investigators[8,97-99] have confirmed the development of primary intraocular melanoma using this method. Specifically, the albino rabbit model of uveal melanoma reproduces the local and systemic behaviour of human uveal melanoma with high efficacy[8]. Since a high number of animals develop metastatic disease, this is a good model to test new therapeutic approaches against primary uveal melanoma and systemic metastases[8].

TUMOUR MARKERS

The discovery of tumour antigens that are recognized by cytolytic T cells, and are able to evoke tumour-specific immune responses in cancer patients has given rise to the possibility of immunotherapeutic treatment. These tumour antigens are classified into three main categories: tumour-specific shared antigens, antigens

encoded by mutated genes, and melanoma-associated antigens. The majority of these antigens are presented to CD8+ T lymphocytes via MHC class I molecules.

Tumour-Specific Shared Antigens. This category contains the MAGE, BAGE, and GAGE genes which are frequently expressed by many tumour types but are silent in normal adult tissue, except testis[100,101]. Sixty percent (60%) of Caucasian cutaneous melanoma patients bear one of the defined antigens encoded by MAGE, BAGE, and GAGE. Immunization against antigens encoded by one of these genes is plausible since male germline cells do not express MHC class I molecules[102,103]. Therefore, gene expression would not result in antigen expression and, in turn, no autoimmune side effects should be noted. Interestingly, however, uveal melanomas were shown to rarely express MAGE genes[104].

Antigens Encoded by Mutated Genes. The study of immunogenic variants of mouse tumours obtained by mutagenesis demonstrated that point mutations in ubiquitously expressed genes could create antigenic epitopes recognized by cytolytic T lymphocytes[105]. In cutaneous melanoma cell lines, point mutations in both the cyclin-dependent kinase 4 gene[106] and the β-catenin gene[107] have produced antigenic peptides presented by HLA-A2 class I molecules. However, since antigens created by point mutations are restricted to very few tumours, it would be difficult to develop cancer vaccines based on these antigens[100,105]. Furthermore, there is no evidence, as yet, of this category of antigens in uveal melanoma.

Melanoma-Associated Antigens. This class includes tyrosinase, gp100/Pmel17, and Melan-A/Mart-1 peptides which are encoded by genes that are frequently expressed in melanoma cells, but are silent in the high majority of normal adult tissues[9]. These melanocytic lineage antigens are presented specifically by HLA-A2 molecules to CD8+ T cells[100]. The T cells can recognize peptides at the tumour cell surface and can subsequently destroy these cells. Hence, immunotherapy trials based upon these melanoma-associated antigens seem feasible. Tyrosinase, gp100/Pmel17, and Melan-A/MART-1 are individually described below.

Tyrosinase. Tyrosinase is an important enzyme in melanin biosynthesis as it converts tyrosine into dihydroxyphenylalanine (DOPA), the precursor of melanin. The tyrosinase gene, so far, encodes for three antigenic peptides in cutaneous melanoma and is expressed in normal and neoplastic melanoma cells, but in no other types of tumours[100].

The implication of tyrosinase as a uveal melanoma-associated antigen has been controversial. Foss and co-workers[108] report that RT-PCR for tyrosinase mRNA detection is of no clinical value in detecting metastatic uveal melanoma. Meanwhile, Tobal and co-workers[109] report that tyrosinase can be detected in uveal melanoma and may be of clinical value. Since tyrosinase expression is highly heterogeneous in cutaneous melanoma[16], it is plausible to suggest that this is also the case in uveal melanoma, as antigenic heterogeneity in uveal melanoma has been reported[110]. This would account for the conflicting reports.

gp100. Gp100 is a premelanosome glycoprotein thought to be specific for melanocytic differentiation[53]. Adema and co-workers[48] also discovered that gp100 RNA transcripts were present in all normal and malignant melanocytic cells analyzed. Brouwenstijn and co-workers[111] later discovered that gp100 transcripts were present in tissues and cell lines other than those of the melanocytic lineage. However, gp100 protein was not detectable. As described earlier, immunohistochemical studies in uveal melanoma have shown gp100 to be highly expressed in this tumour[42,63].

Pmel17 is another antigen arising from the same gene as gp100 antigen through differential splicing. Pmel17 contains an additional 7 amino acids inserted at position 567[47]. Due to Pmel17 high homology with gp100 it is not surprising that HMB-45 also reacts with Pmel17[112,113]. Adoptive transfer of tumour infiltrating lymphocytes directed against gp100/Pmel17 in a cutaneous melanoma patient led to regressions of melanoma metastases.

MART-1/Melan-A. This antigen was discovered separately by two groups of researchers. Due to the melanocytic nature, one group[114] named the gene Melan-A. The other group[115] identified the same cDNA and named it MART-1. Studies[12,13,43,44,45] have shown that MART-1/Melan-A is exclusively specific for normal and malignant melanocytes. Its function remains to be identified, however, it may be involved in melanin synthesis[116].

In the skin MART-1/Melan-A is expressed only in melanocyte-lineage cells[13]. Keratinocytes, Langerhans cells, blood vessels, infiltrating blood cells, sweat glands, and nerve bundles were all found to be negative for the Melan-A antigen. MART-1/Melan-A is the immunodominant MAA recognized by HLA-A2-restricted T lymphocytes in most patients with cutaneous melanoma with HLA-A2 phenotype, which represents 50% of North American whites and Japanese patients with melanoma[13]. Therefore, MART-1/Melan-A may be a useful target of immunotherapy for cutaneous melanoma. However, the heterogeneous expression of MART-1/Melan-A in metastatic cutaneous melanoma may indicate possible tumour escape from T-cell recognition through emergence of antigen-loss variants[13,45]. These facts remain to be elucidated in uveal melanoma.

PATIENTS, MATERIALS, & METHODS

Human Studies

A compilation of 56 uveal melanoma cases spanning a 16-year period, from 1982 – 1998, was made. All uveal melanoma cases were obtained from the Ophthalmic Pathology Registry at McGill University, Montreal Canada. All choroidal and ciliary body cases were obtained after enucleation, while iris tumours underwent iridocyclectomy. The tumours were formalin-fixed and paraffin embedded.

Animal Studies

Our colleagues from the University of Valladolid, Spain, kindly provided the primary and metastatic uveal melanoma tumours from the albino rabbit animal model.

Cell Lines

MKT-BR. The MKT-BR cell line was characterized by R Belkhou and J Sahel[117] (University of Strasbourg, France). It is derived from a primary uveal melanoma tumour obtained from a patient after surgical enucleation[117] and consists of spindle cells with evident cytoplasmic processes[118].

Sp6.5. Pelletier and co-workers[119] (Laval University, Quebec, Canada) established the Sp6.5 cell line from a non-irradiated primary uveal melanoma tumour. The Sp6.5 line exhibits a mixture of spindle and epithelioid cells[118].

92-1. The 92-1 cell line was established from a primary uveal melanoma by De Waard-Siebinga and co-workers[120]. It is composed of highly pleomorphic cells containing large, prominent nucleoli.

Culture Methods

As previously described[118] cells were grown in 25cm^2 tissue culture flasks using RPMI-1640 medium with Glutamax supplemented with 10% heat-inactivated FCS, and 5.0UI/ml-50ug/ml penicillin-streptomycin. The cultures were incubated at 37°C, in a humidified atmosphere of 5% CO_2. The medium was changed every 2-3 days. All reagents were obtained from Life Technologies.

Albino Rabbits

Three groups of male albino New Zealand rabbits weighing around 3.000 g (range: 2.400-3.450 g, mean 2.900 g) treated in accordance with the ARVO statement for the use of animals in Ophthalmic and Vision Research were used. Animals received daily intramuscular (i.m.) Cyclosporin A (CsA) (Sandimun 50 mg/ml; Sandoz, Basel, Switzerland) beginning two days before cell implant at a dosage of 0.3ml/kg for the first 4 weeks and 0.2 ml/kg for the following 4 weeks.

Implant Technique

An aliquot of 0.2 ml from the respective cell suspension (MKT-BR, Sp6.5, 92-1,) with a total amount of 0.36-1x10^6 cells with up to 97% viability was prepared for the choroidal implant.

Ketamine chloridrate *(Ketolar,* Parke-Davis, S.A, Spain) 5 mg/kg of weight i.m and clorhidrate of 2-(2,6-xilidin)-5,6-dihidro-4H-1,3-tiazine (*Rompún 2%*, Bayer AG, Germany) 7 mg/kg of weight i.m. was employed for general anesthesia. Topic mydriatics and anesthetics were also administered. The implantation technique used was a modification of that previously described by Krohn[121]. A temporal conjunctival incision was performed followed by a 0.2 ml paracentesis. Cells were carefully injected in the subchoroidal space using a 25-27 G curve canulae passing through a scleral incision of 3 mm at 6 mm from the limbus.

Follow-up

Rabbits were followed for 2 months. A fundoscopy study, weight, and analytical control was performed weekly. Blood levels of CsA were determined in complete blood with polarized fluorescent immunoanalysis using monoclonal antibodies and an autoanalyzer (Abbot TDX; Abbot, USA).

Postmortem Study

Animals were followed until they spontaneously died. Survival time was considered the last complete week from the beginning of the immunosuppressor treatment. The post-mortem study included a thoracotomy and laparotomy to study intrathoracic and abdominal viscera. Although all the eyes were studied, it was not possible to perform the full autopsy in all the cases. The enucleated eyes and representative fragments of liver and lung were formalin-fixed and paraffin-embedded for histopathological and immunohistochemical evaluation.

Immunohistochemistry

Immunohistochemistry was performed using the peroxidase-anti-peroxidase technique for HMB-45 (1:50; Dako), S-100 protein (1:2000; Dako), and Melan-A (1:100; Novocastra) monoclonal antibodies in formalin-fixed, paraffin-embedded sections. All three antibodies were investigated in the 56 uveal melanoma cases. Melan-A and HMB-45 were examined in the primary and metastatic lesions from the animal model.

Samples were fixed in 10% BNF in a solution that is 15-20X the volume of the tissue. Fixing time was a minimum of 4 hours and a maximum of 24 hours. Sections were loaded onto a Ventana ES immunostaining instrument and each slide was flooded with wash solution from a squeeze bottle. They were then incubated with an inhibitor for 4 minutes at 42°C to remove peroxidase activity. The respective

antibody (Melan-A, HMB-45, or S-100 protein) was applied and incubated for 32 minutes at 42°C, after which the secondary antibody (a cocktail consisting of anti-mouse IgG and IgM; and anti-rabbit IgG) was applied for 8 minutes at 42°C. The avidin HRPO step was performed for 8 minutes followed by DAB solution mixed with H_2O_2 for 8 minutes at 42°C. Finally copper enhancement was performed for 4 minutes at 42°C. Note that at the end of each incubation step, the Ventana ES instrument washed the sections to stop the respective reaction and to remove unbound material that would hinder the desired reaction in subsequent steps.

Grading of the Immunostain

All cases from uveal melanoma patients and the animal model were independently reviewed by two investigators and graded according to the following methods.

Intensity of the Immunostain

The degree of positivity was determined in accordance with other investigators[13,41,62]. A grade of one (1) was given for weak intensity, grade two (2) for moderate intensity, and grade three (3) for strong intensity. A grade of zero (0) was given to all negative tumours.

Percentage of Tumour Area Stained

Cases were graded according to the percentage of tumour area which stained positively, in accordance with other investigators[12,43,44,45]. A grade of zero (0) for negative tumours, grade one (1) for an area between 1 - 25%; grade two (2) for 26 - 50%; grade three (3) for 51 - 75%; and grade four (4) for >75%. When greater than 75% of tumour cells stained positively, the immunostain was said to exhibit an homogeneous staining pattern.

Cell Type Classification

Hemotoxylin and eosin (H&E) stained sections of all human uveal melanoma cases were reviewed in order to classify tumours according to cell type. Each tumour was grouped under one of the following four categories: spindle cell (S); mixed, predominantly spindle cell (MS); mixed, predominantly epithelioid cell (ME); or epithelioid cell (E). Once all the results were tabulated, the respective tumours were matched for cell classification and Melan-A staining.

H&E sections of metastatic uveal melanoma from lung and liver of the albino rabbit were also reviewed for cell morphology, in order to determine if the morphology of a cell line was altered during metastatic progression.

RESULTS

Human Studies

Patient Characteristics

Fifty-six primary uveal melanomas were analyzed by light microscopy. These cases comprised 2 iris melanomas, 2 ciliary body (CB) tumours, 45 choroidal melanomas, and 7 choroidal tumours involving the ciliary body (CCB). Cell type was determined for all tumours through histopathological analysis. There were 13 spindle cell (S); 17 mixed cell, predominantly spindle (MS); 18 mixed cell, predominantly epithelioid (ME); and 8 epithelioid cell (E) tumours. Patient information was collected for only 44 of the 56 cases, as there was no documentation available for 12 of the patients. There were 22 male and 22 female patients involved in the study with a mean age of 63 years. The right eye (OD) was affected in 25 cases and the left eye (OS) in 19 cases. Patient characteristics are summarized in Table 1.

Table 1. Patient Characteristics.

No. Primary Tumours	56
Male/Female*	22 / 22
Mean Age (years)*	63 years (2 not specified)
Range*	43 – 87 years
Location: Iris/CB/Ch/CCB	2 / 2 / 45 / 7
Cell Type	13 S / 17 MS / 18 ME / 8 E
LTD* (average)	14.06mm (2 not specified)
Globe affected OD/OS*	25 / 19

*Information gathered from 44 of the 56 cases
LTD = Largest tumour dimension

Immunostaining of Primary Uveal Melanoma

The level of expression of Melan-A, HMB-45, and S-100 protein in fifty-six uveal melanoma cases are summarized in Tables 2 and 3.

Table 2. Comparison between Melan-A, HMB-45, and S-100 protein according to the intensity of the immunostain in 56 uveal melanoma cases.

Grade of Immunostain	Melan-A	HMB-45	S-100 protein
0	15	3	3
1	18	5	21
2	21	10	23
3	2	38	9

Table 3. Comparison between Melan-A, HMB-45, and S-100 protein according to the percentage of tumour area stained in 56 uveal melanoma cases.

% Tumour Area	Melan-A	HMB-45	S-100 protein
0	15	3	3
1 - 25	8	2	8
26 - 50	3	6	10
51 - 75	11	8	15
> 75	19	37	20

Melan-A exhibited cytoplasmic staining of melanoma cells and was positive in 41 of 56 (73.2%) tumours. The intensity of the stain varied between tumours with an average grade of 1.61 being observed (Figures 6-8). Although the intensity was moderate, Melan-A was found to consistently stain greater than 50% of melanoma cells within a given tumour. Melan-A stained 46.3% of cases in an homogenous fashion.

HMB-45, which also exhibited cytoplasmic staining, was positive in 53 of 56 (94.6%) cases, therefore showing greater positivity than Melan-A. An average intensity of 2.62 was observed for this immunostain (Figures 9-11). In 69.8% of cases, HMB-45 showed homogeneous staining of the tumour.

S-100 protein stained the same number of cases as HMB-45, showing positivity in 53 of 56 (94.6%) cases. It was also shown to stain the cytoplasm of the melanoma cells. However, unlike HMB-45, the average staining intensity of S-100 protein was 1.77, (Figures 12-14) making it comparable to Melan-A. S-100 protein also depicted a more heterogeneous staining pattern than its counterparts. Only 37.7% of cases demonstrated homogeneous staining.

Melan-A Staining According to Cell Type

Melan-A expression did not differ between spindle and epithelioid cells. As depicted in Table 4, grade 3 Melan-A staining was seen in both a MS and an E cell tumour. Nine cases of mainly spindle cells showed grade two staining compared to 12 tumours with mainly epithelioid cells. Grade one staining was observed in 13 mainly spindle cell tumours as compared to 5 mainly epithelioid cell tumours. Finally, the classification of negative staining by cell type was virtually the same, with 7 mainly spindle cell tumours and 8 tumours with mainly epithelioid cells.

Table 4. Correlation between Cell Type and Melan-A staining in 56 uveal melanoma cases.

Melan-A Grading	(S)	(MS)	(ME)	(E)
0	2	5	5	3
1	8	5	3	2
2	3	6	10	2
3	-	1	-	1

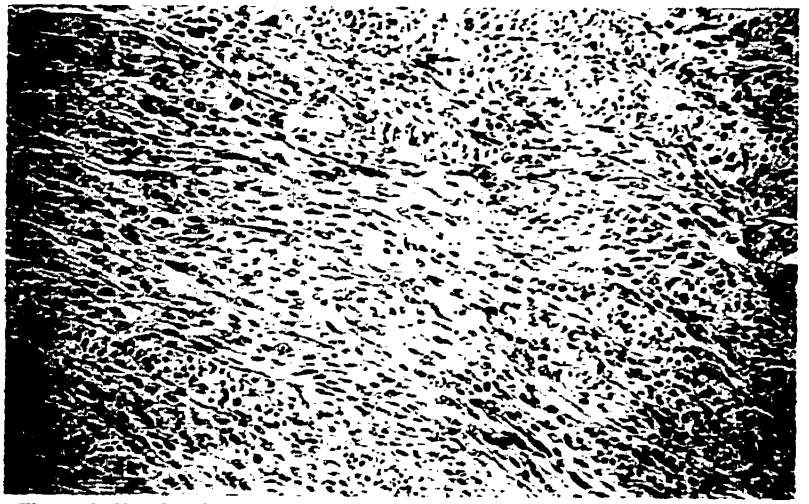

Figure 6. Uveal melanoma depicting grade one positivity for Melan-A. (1108X)

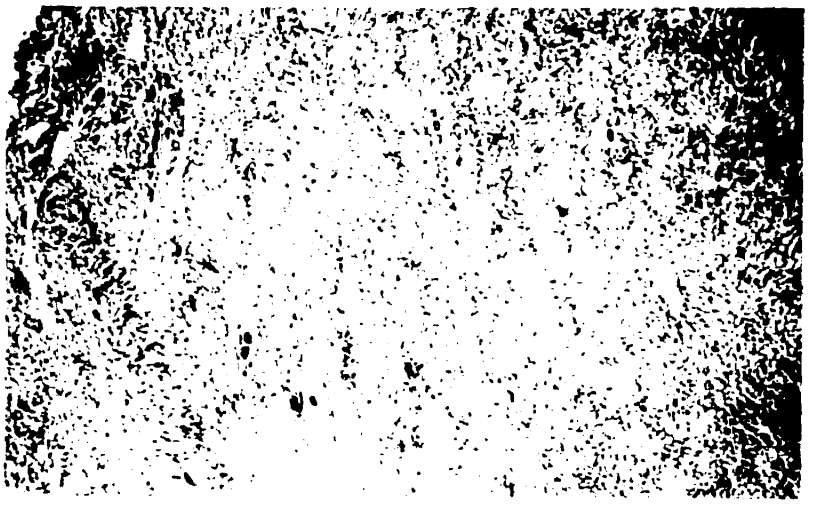

Figure 7. Uveal melanoma depicting grade two positivity for Melan-A. (1108X)

Figure 8. Uveal melanoma depicting grade three positivity for Melan-A. (1108X)

Figure 9. Uveal melanoma depicting grade one positivity for HMB-45. (1108X)

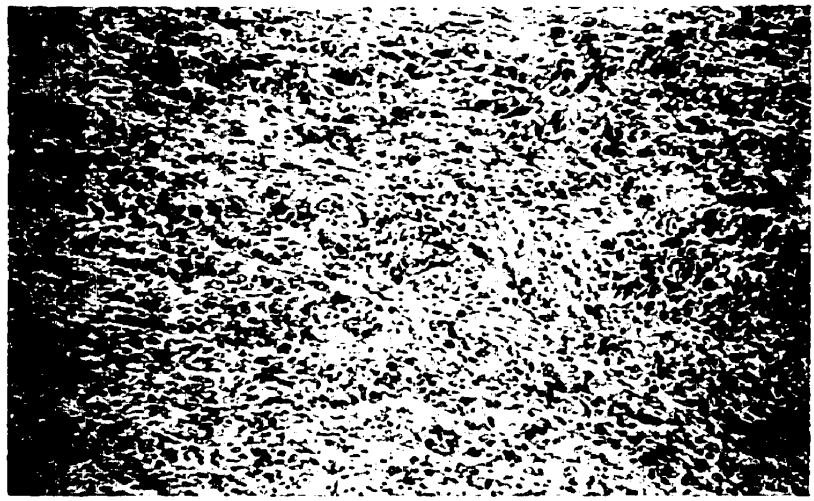

Figure 10. Uveal melanoma depicting grade two positivity for HMB-45. (1108X)

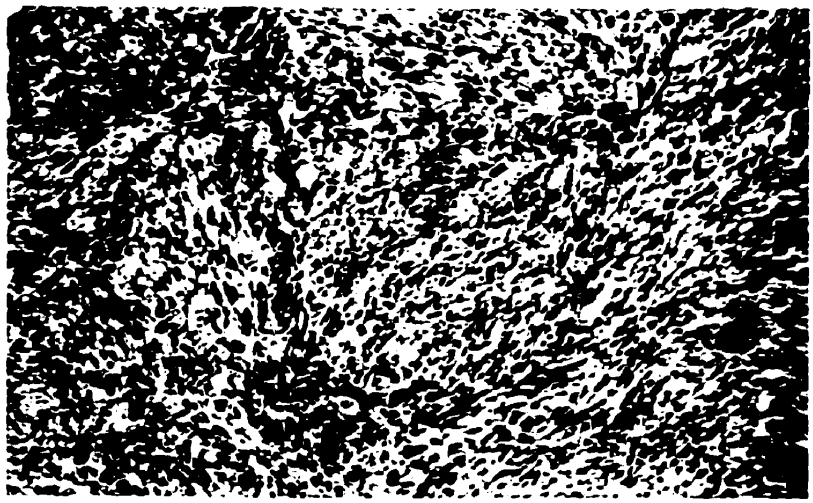

Figure 11. Uveal melanoma depicting grade three positivity for HMB-45. (1108X)

Figure 12. Uveal melanoma depicting grade one positivity for S-100 Protein. (1108X)

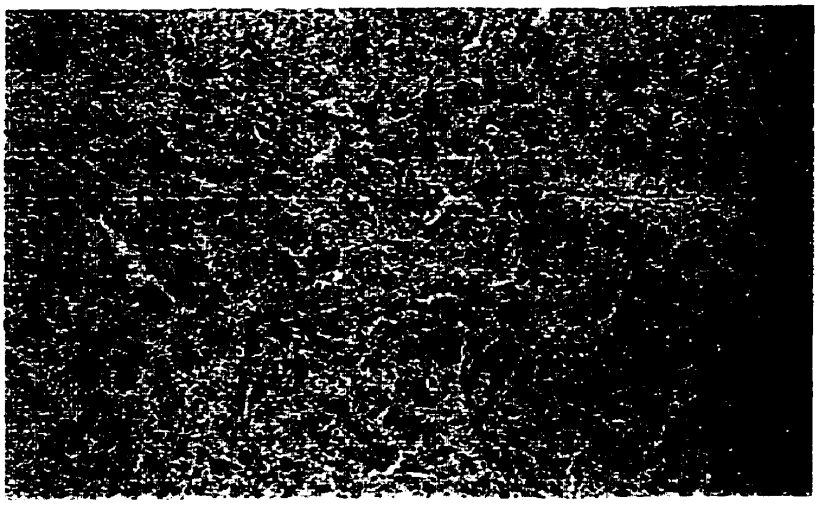

Figure 13. Uveal melanoma depicting grade two positivity for S-100 Protein. (1108X)

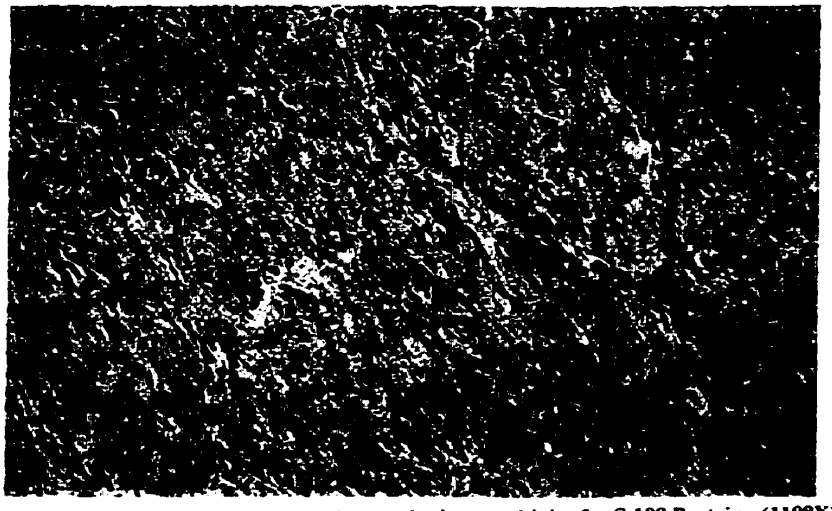

Figure 14. Uveal melanoma depicting grade three positivity for S-100 Protein. (1108X)

Animal Studies

Albino Rabbit Characteristics

Twenty-two albino rabbits were involved in the study. There were 3 rabbits implanted with the MKT-BR cell line; 10 with 92-1; and 9 with Sp6.5. All rabbits developed large, posterior choroidal melanoma tumours. Both lung and liver metastases were observed in 2 rabbits, while 14 rabbits had lung metastases only, and 2 rabbits had only liver metastases. There was no evidence of metastatic disease in 4 rabbits. As described in the methods, MKT-BR, 92-1, and Sp6.5 exhibit different cell morphologies, however, the cell morphology observed in the metastatic nodules was predominantly epithelioid in all cases, regardless of the cell line. There were 4 rabbits that did not develop any metastatic disease. These characteristics are summarized in Table 5.

Table 5. Tumour Characteristics for 22 Albino Rabbits.

Cell Line	Primary Tumour Cell Morphology	Number of Rabbits with a Primary Tumour	Number of Rabbits with Metastases		
			Lung	Liver	Both
MKT-BR	S	3	1	-	
Sp6.5	ME	9	6	2	1
92-1	ME	10	7	-	1

Immunostaining of Primary Uveal Melanoma

The three cell lines were positive for both Melan-A and HMB-45 immunostains, since all 22 primary tumours stained. The graded intensity and the

percentage of cells stained are summarized in Table 6 and Table 7, respectively (Figures 15-16).

Table 6. Staining intensity of Melan-A and HMB-45, respectively, in 22 cases of uveal melanoma developed in the albino rabbit.

Grade	Melan-A	HMB-45
0	-	-
1	3	-
2	4	1
3	15	21

The average staining intensity observed for Melan-A was 2.55, while HMB-45 exhibited an average intensity of 2.95. Melan-A stained less than 50% of cells within a given tumour in 81.8% of cases, thereby demonstrating a heterogeneous staining pattern. Melan-A stained greater than 50% of cells in only 18% of cases. However, HMB-45 showed a more homogeneous staining pattern since more than 50% of cells were positive in 72.7% of cases.

Table 7. Percentage of tumour cells stained by Melan-A and HMB-45, respectively, in 22 cases of uveal melanoma developed in the albino rabbit.

% Tumour Cells	Melan-A	HMB-45
1 - 25	4	-
26 - 50	14	6
51 - 75	-	6
> 75	4	10

Figure 15. Primary uveal melanoma tumour developed in the albino rabbit, showing positivity for HMB-45. (692X)

Figure 16. Primary uveal melanoma tumour developed in the albino rabbit, showing positivity for Melan-A. (692X)

Immunostaining of Metastatic Uveal Melanoma

Nodules of cells were observed in all cases of lung metastases, while more diffuse metastases was observed in the liver. Melan-A and HMB-45 staining was positive in all cases (Figures 17-19). The results are shown in Table 8.

Table 8. Level of expression of HMB-45 and Melan-A in 16 cases of lung and 4 cases of liver metastases from the albino rabbit

Grade of Immunostain	Melan-A		HMB-45	
	Lung	Liver	Lung	Liver
0	-	-	-	-
1	6	1	3	1
2	5	3	5	1
3	5	-	8	2

Again, a stronger staining intensity was observed with HMB-45 as compared to Melan-A. The average intensity of HMB-45 was 2.31 in lung metastases and 2.25 in liver metastases. Melan-A exhibited an average intensity of 1.94 in lung metastases and 1.75 in liver metastases.

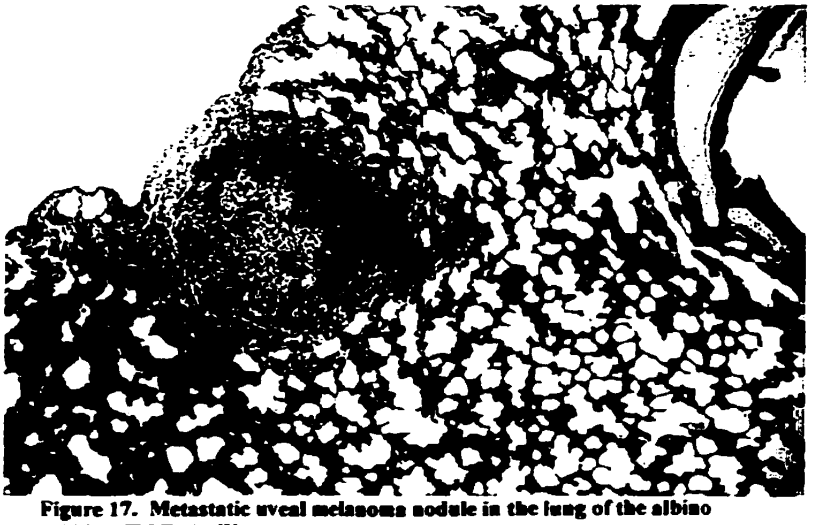

Figure 17. Metastatic uveal melanoma nodule in the lung of the albino rabbit. (H&E; 346X)

Figure 18. Metastatic nodule staining for HMB-45. (1385X)

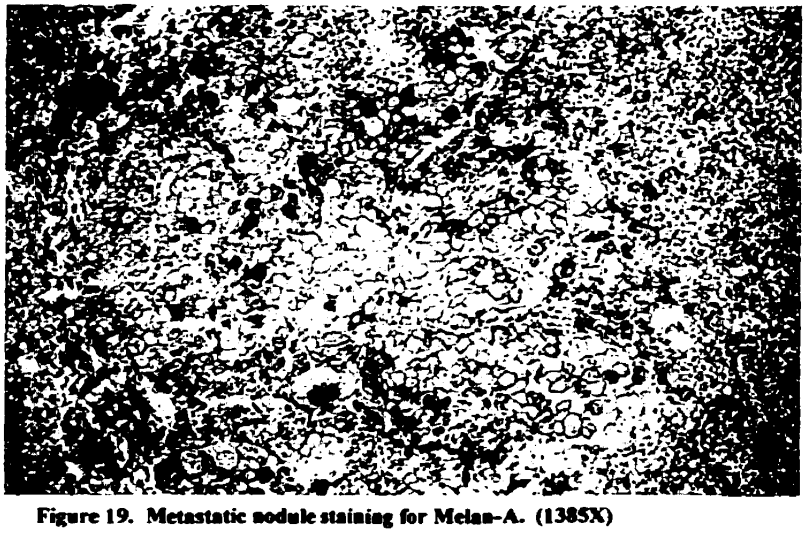

Figure 19. Metastatic nodule staining for Melan-A. (1385X)

DISCUSSION

The recent literature[10-16,43,44,45] has spotlighted on MART-1/Melan-A antigen as a good immunohistochemical and mRNA marker for cutaneous melanoma. Consequently, MART-1/Melan-A antigen was chosen to be investigated as an immunomarker for uveal melanoma. This is the first study to examine MART-1/Melan-A expression in a large series of paraffin-embedded primary uveal melanomas. It is also the first study to examine melanoma-associated antigen expression in primary and metastatic human uveal melanomas developed in albino rabbits.

Human Studies

Melan-A was found to be positive in 73.2% of primary uveal melanoma tumours. This finding contradicts a recent immunohistochemical study[44] of Melan-A in 12 cryostat sections of ocular melanomas, which found Melan-A to be positive in only 25% of cases. The latter low percentage may be due to the small sample size used. Interestingly, de Vries and co-workers[46] report positivity of Melan-A in 100% of frozen uveal melanoma sections analyzed. It may therefore be possible that there is a higher reactivity of the antibody in frozen sections as compared to paraffin-embedded cases. Several studies[13,64] have reported lower reactivity of HMB-45, S-100 protein, and Melan-A in paraffin-embedded sections than in frozen sections. However, there is the possibility of false-negative results in paraffin-embedded tissues since some antigens do not survive fixation and embedding procedures. As well, specimens may lose antigenicity over time, therefore older tissues may not react as well as newer ones. Conversely, false-positive results may be a problem in the

case of S-100 protein due to the lack of specificity of this antibody[42,64]. Although HMB-45 has recently been described to be positive in non-melanocytic tumours outside of the eye[56-60], it was not found to stain non-melanocytic ocular tumours[42,51] and remains to be very specific for melanocytes. Melan-A is also very specific for melanocytes[12,13,43,44,45]. Therefore, no false-positive results should be seen for these two antibodies. It is, however, important to emphasize that paraffin-embedded sections represent a permanent record of cases, which can be examined over a long period of time, therefore, allowing for the investigation of a larger series of cases.

HMB-45 and S-100 protein were found to be positive in 94% of uveal melanomas. These results are consistent with the literature[42,51,63]. Steuhl and co-workers[51] found S-100 protein to be positive in 91% of uveal melanomas and HMB-45 in more than 95% of uveal melanomas. Martins and co-workers[63] found HMB-45 to be positive in 100% of cases and S-100 in 75% of ocular melanomas.

In accordance with other investigators[14,42,63] a grading system from 0 to 3, denoting negative to strong staining, was used to determine the intensity of the immunohistochemical reaction. Thus providing an effective qualitative analysis of all specimens. The intensity of the immunostain was strongest with HMB-45, while Melan-A and S-100 protein showed similar staining intensity. The reasons for variable intensity are not well defined. Possibly, antigen expression is decreased in some cells as opposed to others. Thus, less antibody binds and the intensity is weaker. Another possibility may be that the antibody is not binding sufficiently

during the immunohistochemical procedure, causing a weaker intensity to be observed. It can be noted that the use of all three markers combined will allow an increased number of positive cases to be detected, as all cases that stained negatively for one of the immunomarkers, showed positivity for at least one other marker.

Also, in accordance with other investigators[13,44,45,46], a grading system based on the percentage of tumour cells staining positively is applied to determine focal versus diffuse staining of each tumour. This allows for the quantitative analysis of the immunostaining. Melan-A was found to stain greater than 50% of melanoma cells in 73.2% of cases. Therefore, Melan-A has a moderately diffuse staining pattern for uveal melanoma. Conversely, Melan-A was found to stain primary cutaneous lesions homogeneously[13]. HMB-45 stained 69.8% of uveal melanoma cases homogeneously while S-100 protein depicted an homogeneous staining pattern in 37.7% of tumours. Interestingly, de Vries and co-workers[46] found that greater than 75% of tumour cells were positive for Melan-A and HMB-45 in 80% of frozen sections investigated. Again this may be due to a higher reactivity seen in frozen sections or may reflect the sample population. While HMB-45 remains the best immunomarker for uveal melanoma, the same is not true for cutaneous melanoma. Blessing and co-workers[12] report that HMB-45 sensitivity is poor with patchy staining. Melan-A is not as intensely expressed in uveal melanoma, as compared to cutaneous melanoma. Melan-A stains both benign and melanocytic cutaneous lesions in a very similar fashion to S100 protein, and more reliably than HMB-45[12].

Cell type and tumour size are the most important prognostic factors for uveal melanoma[4,29]. Since it is impossible to determine time of metastasis, it would be helpful to have a diagnostic indicator to define patients who are at highest risk for developing metastases. This study examined the role of Melan-A in this context in the hopes that its staining pattern among different cell types would be of prognostic significance. However, there was no correlation found between Melan-A staining and cell type in this study. Melan-A is expressed in a range of tumours, from well to poorly differentiated, and of different cell types. Therefore, Melan-A staining does not correlate with prognosis.

It can be concluded that Melan-A is adequately expressed in uveal melanoma tumours since it is positive in 73.2% of paraffin-embedded sections and exhibits a moderately diffuse staining pattern. As mentioned, HMB-45 remains the best immunomarker for uveal melanoma, however, Melan-A antibody can be included in the immunohistochemical profile of uveal melanoma.

Animal Studies

The albino rabbit animal model, which is developed using human uveal melanoma cell lines, was used to study antigen expression in metastatic lesions, due to the difficulty of obtaining metastatic lesions from uveal melanoma patients. Antigen expression in the primary uveal melanoma tumour developed in the albino rabbit was also studied and compared with the findings obtained in the human studies section of this report.

Melan-A and HMB-45 immunostains were investigated in primary and metastatic uveal melanoma lesions developed in the albino rabbit, since they specifically recognize melanoma-associated antigens Melan-A and gp100, respectively. It is therefore possible to assess Melan-A and gp100 expression in the cases examined.

Although S-100 protein is a good immunohistochemical marker for uveal melanoma, it will not be considered in the group of possible immune targets due to its highly expressive nature in many tissues. Significantly lower staining for S-100 has been reported in metastatic uveal melanoma and S-100 is less reliable in the diagnosis of difficult cases of undifferentiated metastases[88].

HMB-45 and Melan-A were positive in all primary and metastatic uveal melanoma lesions developed in the albino rabbit. HMB-45 stained more intensely and more homogeneously in the primary tumour than seen for Melan-A staining. This is consistent with the results obtained in the human studies section of this report. Eventhough both HMB-45 and Melan-A stained all metastatic lesions diffusely, HMB-45 showed a stronger staining intensity than Melan-A. The finding for HMB-45 is in accordance with studies performed on human specimens. In a study of 29 primary uveal melanoma tumours with corresponding metastases, Luyten and co-workers[88] found that HMB-45 was the most sensitive immunohistochemical marker for the analysis of metastatic uveal melanoma. The most aggressive cell types kept

their immunoreactivity for HMB-45[88]. In contrast, HMB-45 showed negative and focal staining in cutaneous metastatic lesions[64].

A more heterogeneous staining pattern was seen for Melan-A in the primary uveal melanoma tumours developed in the albino rabbit as compared to the human studies. Melan-A stained less than 50% of melanoma cells in 81.8% of cases. However, Melan-A was found to stain all metastatic lesions in an homogeneous fashion. In contrast, Melan-A was found to stain primary cutaneous tumours homogeneously, and metastatic lesions in a heterogeneous fashion[13].

HMB-45 exhibited a more homogeneous staining pattern in the primary tumour than Melan-A, yet both depicted homogeneous staining patterns in metastatic lesions. Interestingly, an increase in both Melan-A and gp100 antigen expression is seen during metastatic progression of uveal melanoma. However, de Vries and co-workers[46] report a lower expression of these antigens in 4 metastatic uveal melanoma lesions analyzed. Due to the small number of lesions examined, this finding may not be significant. In fact, de Vries and co-workers[46] suggest that further examination of these antigens in metastatic uveal melanoma lesions is warranted. The findings in this present study are opposite to results in cutaneous melanoma. Kageshita and co-workers[13] discovered that gp100 and Melan-A antigen expression is decreased with disease progression, while Marincola and co-workers[45] found that less than 50% of metastatic cutaneous lesions stained for Melan-A. The loss of melanoma-associated antigen expression in cutaneous melanoma suggests that downregulation may occur in vivo and could contribute to tumour escape from immune recognition[45]. Is it

possible that melanoma-associated antigens in uveal melanoma are not downregulated because the immune system has not been primed to fight against these cells? In such a case, it may seem plausible to suggest that the cells would not need to utilize immune escape mechanisms, thereby owing to the high antigen expression on these cells.

The increase in antigen expression during disease progression of uveal melanoma is a significant finding since antigens chosen as targets for immunotherapy must be adequately expressed on tumour cells. It can be concluded that melanoma-associated antigens, Melan-A and gp100, are adequately expressed in the metastatic uveal melanoma lesion and may be excellent candidates as tumour targets for immunotherapeutic strategies. Furthermore, since both Melan-A and gp100 antigens are expressed by the primary and metastatic lesion, it can be hypothesized that these same antigens are present on the cells while they undergo metastatic dissemination. Melan-A and gp100 antigens would therefore be effective tumour targets to detect circulating malignant cells in uveal melanoma patients.

It is interesting to examine the morphology of tumour cells in the primary lesion versus the metastatic site. For instance, the MKT-BR cell line exhibits mainly spindle cells in the primary tumour and mainly epithelioid cells in the metastatic lesion. Do different cell lines produce metastases? Do spindle cell lines have fewer metastases than epithelioid cell lines? Can cells differentiate outside their place of origin? MKT-BR cell line does not develop metastases as efficiently as more poorly

differentiated cell lines. The Sp6.5 cell line, which is composed of highly pleomorphic epithelioid cells, was found to more efficiently produce ocular tumours and metastatic disease[8]. Different cell lines allow early to late stage disease to be examined and support the idea that epithelioid cells are more strongly associated with metastatic disease than spindle cells. A significant higher percentage of epithelioid cells was found in metastatic uveal melanoma compared with the primary melanoma[88]. This finding is consistent with our animal studies, in which all metastases were comprised of a majority of epithelioid cells.

Future Implications on Clinical Management of Uveal Melanoma

Since MART-1/Melan-A and gp100 mRNA are detectable in patients with cutaneous melanoma[15-19], and it is now known that uveal melanoma cells express these antigens, then we suspect that they will be good markers to detect circulating malignant cells in uveal melanoma patients. Consistent with other reports[12,13], this study provides early experimental data to support the use of melanoma-associated antigen-related peptides in the vaccination of melanoma patients. Coulie and co-workers[114] found Melan-A to be expressed in 100% of cutaneous melanoma lesions through RT-PCR technique. Chen and co-workers[122] determined Melan-A expression through immunohistochemical analysis and RT-PCR for mRNA expression. They found that 16 of 17 cutaneous melanoma lesions expressed both Melan-A mRNA and positive immunostaining.

Staining patterns have to be interpreted in the full knowledge of their potential limitations[12]. In order to develop successful immunotherapeutic strategies, it is important to carefully map the melanoma antigens by immunohistochemistry on biopsy material from the melanoma patient before treatment is started[11]. Although this method is accessible for cutaneous melanoma, it is impossible for uveal melanoma unless the eye is enucleated. If metastasis is possible in small and medium sized tumours, cases in which the eye is not enucleated, how can antigen expression be assessed? Detection of circulating malignant cells is the key.

Why are these issues so important for uveal melanoma? It is impossible to define the exact time when cells metastasize and to follow metastatic progression in uveal melanoma. Do only epithelioid cells metastasize? This would seem to be the case since all metastatic lesions from our animal studies were composed of epithelioid cells. However, it may be possible that spindle cells are capable of metastasizing and transforming during dissemination, thus owing to the epithelioid population in the metastatic lesion. This would suggest that small tumours composed of spindle cells might be able to undergo metastases as well as larger tumours. To further complicate issues, what are these cells, which undergo hematogeneous dissemination, doing for an average of 6.5 years? Do they remain in the circulation or do the cells plant themselves in the liver waiting for an opportune time to grow? Since metastatic disease is undetectable until the liver is affected, there is a need to focus on the detection of subclinical metastases, namely circulating malignant cells. This early

detection of tumour cells may provide a means of preventing clinical metastases from occurring altogether, by altering treatment for the patient.

The Importance of Immunotherapy for Uveal Melanoma

Metastasis from uveal melanoma occurs on average 6.5 years after removal of the primary tumour[6]. Uveal melanoma cells have an affinity for the liver, and once hepatic metastasis is detected, life expectancy for the patient is between two to seven months and fatal in all cases[88]. There is no staging for uveal melanoma to give warning signals. It can be likened to a game of Russian roulette. It is pertinent that research be directed towards treatment of metastatic disease and most importantly, towards prevention of hepatic involvement. For these reasons, the ability to detect circulating malignant cells is crucial to the survival of uveal melanoma patients.

Uveal melanoma represents an almost unique tumour in having histopathologically distinguishable sub-populations of cells with differing metastatic capabilities. Spindle cells are rarely found in metastases, whereas epithelioid cells, with their lack of cohesiveness, have a high potential to metastasize[4]. If viable malignant cells naturally selected for their metastatic potential are circulating, their detection in patients with a negative metastatic work-up could initiate treatment such as chemotherapy that could pre-empt metastasis formation and may even, therefore, be curative for the patient once the primary lesion has been removed.

Since we do not normally expect melanocytes to be present in the peripheral circulation, the detection of MAA mRNA is a suitable indicator of the presence of circulating melanocytes[18]. In accordance with previous studies in cutaneous melanoma[15,17] which apply a multiple marker assay to account for heterogeneity, a multiple marker assay including both MART-1/Melan-A and gp100 could increase the detection of circulating uveal melanoma cells. It may be possible that while treatment is localized to the primary tumour, there are already viable cells circulating in the peripheral blood. These cells are not affected by treatment and go on to form metastatic lesions years after surgery.

In detecting circulating malignant cells in uveal melanoma patients who do not have metastatic disease, it may be possible to classify these patients into two treatment groups independent of their ocular tumour. That is to say, that for patients without metastasis and in whom no circulating malignant cells are present, local treatment is the treatment of choice. However, if circulating malignant cells are identified in some patients whom do not have metastatic spread then an alternate form of treatment will be administered. Hence, the detection of tumour dissemination at an early stage, rather than detection of metastasis, would be of clinical benefit to the patient.

CONCLUSIONS

Human Studies

1. mAb Melan-A merits inclusion in the immunohistochemical profile of uveal melanoma.

2. mAb Melan-A does not correlate with prognosis for uveal melanoma.

Animal Studies

1. mAbs Melan-A and HMB-45 are positive in all primary and metastatic uveal melanoma lesions.

2. In contrast to cutaneous melanoma, melanoma-associated antigens, Melan-A and gp100, are upregulated during disease progression in uveal melanoma.

3. Melan-A and gp100 antigens may be effective immunomarkers to detect circulating malignant cells and for potential immunotherapeutic techniques in uveal melanoma. Studies in these areas should be investigated.

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