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Characterization of ocular and metastatic
uveal melanoma in an animal model

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of the degree of Master of Science

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Abstract

Uveal melanoma is the most common primary malignant intraocular tumor in adults. The management of uveal melanoma remains a clinical dilemma, which reflects our poor understanding of this life-threatening disease. A major challenge facing researchers investigating this malignancy has been to develop a suitable animal model. The purpose of this work is to characterize, in detail, the processes of tumor development, malignant cell dissemination and metastasis in a 10-week albino rabbit model of uveal melanoma. Intraocular tumors successfully developed, and metastatic disease was present in all animals at the end of the experiment. For the first time using an animal model of uveal melanoma, the presence of circulating malignant cells in the bloodstream was demonstrated. Knowledge gained from this study has led to a better overall understanding of the progression of the disease in this experimental model and may facilitate the development of methods for the prevention, early detection and treatment of metastatic uveal melanoma.

Résumé

Le mélanome de l'uvée est la tumeur primaire maligne intraoculaire la plus répandue chez les adultes. Le contrôle de ce type de mélanome demeure un dilemme clinique, reflétant notre manque de connaissance de cette maladie potentiellement mortelle. L'un des plus grands défis pour les chercheurs travaillant sur cette maladie, est de développer un modèle animal adéquat. L'objectif de cette étude était de caractériser en détail le processus de développement de la tumeur, la propagation des cellules malignes et les métastases chez le modèle de 10 semaines du mélanome de l'uvée du lapin albinos. Les tumeurs intraoculaires se sont développées avec succès, et la maladie métastatique était présente chez tous les animaux à la fin de l'expérience. Pour la première fois, la présence de cellules malignes dans la circulation sanguine, a été démontrée en utilisant ce modèle. Les connaissances acquises durant cette étude ont permis une meilleure compréhension générale de la progression de la maladie chez ce modèle expérimental et offrent la possibilité de faciliter le développement de méthodes de prévention, comme la détection précoce et le traitement du mélanome métastatique de l'uvée.

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List of abbreviations

AEC	3-amino-9-ethylcarbazole
Bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
CMC	Circulating malignant cells
CsA	Cyclosporin A
dATP	Deoxy adenosine triphosphate
dCTP	Deoxy cytosine triphosphate
dGTP	Deoxy guanosine triphosphate
DMBA	7,12-dimethyl-benz[a]anthracene
DNA	Deoxyribonucleic acid
DNAse I	Deoxyribonuclease I
dNTP	Deoxy nucleotide triphosphate
DTT	Dithiothreitol
dTTP	Deoxy thymidine triphosphate
EDTA	Ethylenediamine tetra-acetic acid
FBS	Fetal bovine serum
FeLV	Feline Leukemia virus
FeSV	Feline Sarcoma Virus
FNAB	Fine needle aspiration biopsy
H&E	Hematoxylin and Eosin
HPF	High power field
IL-2	Interleukin 2
LFT	Liver function tests
LTD	Largest tumor dimension
mAb	Monoclonal antibody
MMLV-RT	Moloney Murine Leukemia Virus-Reverse Transcriptase
MTLN1	Mean of the Ten Largest Nucleoli
°C	Degrees Celsius

PAS	Periodic Acid Schiff
PET	Positron emission tomography
PBS	Phosphate Buffered Saline
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute media
RT	Room temperature
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
Taq	Thermus aquaticus
TBS	Tris buffered saline
TIL	Tumor infiltrating lymphocytes
U	Units

Chapter 1
INTRODUCTION

Uveal melanoma has received a great deal of research attention due to its potential to jeopardize not only the vision but also the life of patients. Although rare, uveal melanoma is the most common primary malignant intraocular tumor in adults. The significant advances in the ability to diagnose the primary tumor have not been accompanied by improvements in the survival rate. Metastasis, the leading cause of death among patients with uveal melanoma, can only be diagnosed at late stages of the disease, when no effective treatment is available. Since less than 2% of the patients present clinically evident disseminated disease at the time of diagnosis (De Croock and Verbraeken 2002), most patients receive only local treatment. Nevertheless, life expectancy is independent of successful local treatment, which suggests that the disease was already disseminated at the time of diagnosis (Shields, Shields et al. 1991), either as circulating malignant cells (CMC) or as occult micrometastatic lesions. Mortality rates are still alarming, with more than 40% of patients progressing from local to systemic disease and dying within 10 years of diagnosis (COMS 2001). Unfortunately, currently available screening tests aimed at detecting metastatic disease at an early stage are of limited value. Once metastasis is diagnosed, life expectancy is reduced to less than 1 year (Albert, Niffenegger et al. 1992). Treatment options for patients with clinically disseminated disease are scarce and usually unsuccessful.

The low incidence of uveal melanoma and the delayed clinical detection of metastasis complicate clinical studies of this disease. Pathological evaluation of enucleated eyes has become rare owing to the tendency towards conservative local treatments. In addition, autopsy rates have decreased, limiting the availability of metastatic tissues for the study of systemic spread. For these reasons, the establishment of

animal models of uveal melanoma is critical not only to better understand the natural course of the disease but also to evaluate new diagnostic and therapeutic strategies.

During the past few decades, several animal models have been used for the study of uveal melanoma. In orthotopic tumor models, melanoma cells are inoculated into the eye of a host animal to induce tumor formation. Orthotopic models are preferred for the study of disease progression, since tumors that develop in their natural microenvironment will present similar biological and metastatic properties to those observed in the clinical setting.

Animal models of uveal melanoma have been developed using melanoma cells derived from three different origins: mouse, hamster and human. The mouse B16 melanoma cell line was primarily used in its natural host for the study of metastasis and the immunological response to the disease (Nieder Korn 1984; Yokoyama, Yoshie et al. 1989). The hamster Greene melanoma cell line was broadly employed to conduct pre-clinical research in rabbits (Fournier, Saulenas et al. 1985; Skov, Kumi et al. 1987; Olsen, Blumenkranz et al. 1988; Ozler, Nelson et al. 1992; Yang, Olsen et al. 1993; Schmidt-Erfurth, Flotte et al. 1996). Both the Greene and B16 melanoma cell lines present the disadvantage of being not only of animal origin but also of cutaneous origin. As a result, the biological behavior of the tumors generated by these two cell lines may differ to some extent from that observed with human uveal melanoma cells (Dithmar, Albert et al. 2000; Blanco, Caissie et al. 2004).

The use of human uveal melanoma cell lines was introduced for the development of improved experimental models. Animal models were developed by injecting human uveal melanoma cells into the eyes of immunodeficient hosts such as athymic nude mice (Albert, Wagoner et al. 1980) and immunosuppressed rabbits (Kan-Mitchell, Mitchell et al. 1989). The main advantage of using rabbits is their larger eye size, which permits both a more accurate injection of the cell suspension in the posterior compartment and allows for the use of conventional diagnostic techniques for tumor follow up. This was the model selected for our studies since it combines the advantages of being orthotopic and using both human uveal melanoma cells and rabbits.

Since its establishment in 1989 (Kan-Mitchell, Mitchell et al. 1989), the model has been successfully used in a variety of studies (Mueller, Folberg et al. 1999; Ozerdem, Mach-Hofacre et al. 2002). However, the time-course of the progression of uveal melanoma following the inoculation of human uveal melanoma cells into the rabbit eye has not yet been described in detail. Understanding the natural history of uveal melanoma in this model is essential for the design of suitable pre-clinical studies. Only a thorough characterization of the model will allow for the correct interpretation of the changes produced by new therapies.

Chapter 2
LITERATURE REVIEW

2.1 Eye anatomy

The eye is the peripheral organ of vision. It is a complex optical system composed of three layers: the outer or supportive coat, the middle or vascular coat and the inner or neural coat.

The supportive coat is represented by the sclera and cornea. The sclera is a dense, white, fibrous tissue that covers the posterior five-sixths of the eye, providing support and protection to the eyeball while maintaining its shape. The visible part of the sclera is covered by a thin membrane called the conjunctiva. The cornea, which merges with the sclera at a region known as the limbus, is a clear, transparent structure that covers the central portion of the anterior surface of the eye. It has the most optical power of the eye and, together with the lens, helps to properly focus the incoming light onto the retina. The vitreous cavity, located between the lens and the retina, is filled with a clear, jelly-like substance called vitreous humor that allows light to pass through.

The middle coat is composed of the uveal tract, which is a pigmented, vascular layer consisting of three structures: iris, ciliary body, and choroid. The iris is the most anterior part of the uvea lying behind the cornea and in front of the lens. It is a ring-shaped tissue that determines eye colour and is responsible for regulating the amount of light that enters the eye by acting as a diaphragm, adjusting the size of the pupil according to the ambient light. The ciliary body, continuous with the iris anteriorly and with the choroid posteriorly, is composed of the ciliary muscle and the ciliary processes. The former participates in the act of accommodation and the latter are responsible for the secretion of aqueous humor, a clear fluid that fills the anterior and posterior chambers.

The choroid is located between the sclera and the retina. It contains numerous blood vessels and its blood supply is considered as one of the highest in the human body. The choroid provides nutritional support to the outer layers of the retina, from which it is separated by the Bruch's membrane. The suprachoroidal space lies between the choroid and the sclera. It is a virtual space that, due to the loose collagen bonds between the two structures, can easily become a real space. Despite being avascular in nature, the suprachoroidal space is crossed by several vessels.

The retina, part of the central nervous system, is the innermost coat that lines the posterior two-thirds of the eye. It is a multi-layered sensory tissue that contains photoreceptors that capture light rays and convert them into electrical impulses. These impulses are transmitted along the optic nerve to the brain, where they are translated into visual images. The macula, located in the centre of the retina, is an area with a high concentration of photoreceptors. It contains the fovea, which is the site of the sharpest vision.

2.2 Uveal melanoma

2.2.1 Epidemiology

Uveal melanoma is a rare malignancy with an estimated incidence of 6 cases per million (Egan, Seddon et al. 1988; Singh and Topham 2003). However, it is the most common primary malignant intraocular tumor in adults and the second most common type of melanoma after skin, representing 5.2% of all melanomas (Chang, Karnell et al. 1998). Uveal melanoma accounts for 85% of ocular melanomas, followed by conjunctival melanoma with 4.8% of the cases (Chang, Karnell et al. 1998). During the past several decades an increase in the frequency of melanoma has been observed. This increase is mainly attributed to an increase in cases of cutaneous melanoma, while the incidence of uveal melanoma has remained unchanged (Egan, Seddon et al. 1988; Singh and Topham 2003).

2.2.2 Risk factors

Although the etiology of uveal melanoma is unknown, several risk factors have been recognized.

Age

The risk of uveal melanoma increases with age. The mean age at diagnosis is 55 years. The disease is rare among children and adolescents. Less than 1% of the patients are under 20 years old at presentation (Singh, Shields et al. 2000). Congenital uveal melanoma is extremely uncommon (Broadway, Lang et al. 1991).

Gender

Men have a slightly higher incidence of uveal melanoma than women (Egan, Seddon et al. 1988).

Race

Uveal melanoma is more common among Caucasians. It is estimated that Caucasians have eight times the probability of developing uveal melanoma compared to Blacks and 3 times greater a probability than Asians (Egan, Seddon et al. 1988). Among Caucasians, people with blonde hair and a blue iris carry the highest risk (Saornil 2004).

Genetic factors

The majority of cases of uveal melanoma are sporadic. Less than 1% arises in patients with a family history of the disease (Singh, Shields et al. 1996). Uveal melanoma is rarely bilateral. However, the incidence of patients with familial or bilateral disease is greater than predicted by chance, suggesting a genetic predisposition. Indeed, associations between uveal melanoma and genetic alterations such as monosomy of chromosome 3, trisomy of chromosome 8 and alterations in chromosome 6 have been reported (Prescher, Bornfeld et al. 1996; Sisley, Parsons et al. 2000).

Sun exposure

Since uveal melanoma develops from melanocytes and it is more common among white fair-skinned patients, sunlight was proposed as a risk factor (Holly, Aston et al. 1990). However, no parallel increase in the incidence of uveal and skin melanoma was

observed during the last decades, as would be expected. Therefore, the role of ultraviolet light in the pathogenesis of uveal melanoma is not as clear as for skin melanoma.

Predisposing conditions

Nevi, ocular and oculodermal melanocytosis (nevus of Ota) are predisposing factors for uveal melanoma. Although choroidal nevi are common (1-6% of the population), these lesions rarely undergo malignant transformation (Ganley and Comstock 1973). On the other hand, it is believed that most uveal melanomas develop from pre-existing nevi (Hungerford 1995). Nevus of Ota is a congenital condition in which the uvea and episclera are hyperpigmented. This is the most common condition that precedes the development of the tumor and increases the risk for uveal melanoma by 30 times (Singh, De Potter et al. 1998).

2.2.3 Clinical features

Uveal melanoma may arise from melanocytes located in the iris (anterior uveal melanoma), the ciliary body and the choroid (posterior uveal melanomas).

Most uveal melanomas develop in the choroid (80% of the cases) (Hungerford 1995). Although some patients with choroidal melanoma initially experience painless visual loss usually due to associated retinal detachment, most patients are asymptomatic and are diagnosed during routine ophthalmoscopic examination. In cases of delayed diagnosis, patients may complain of pain due to inflammation or secondary glaucoma. As the tumor grows it may rupture the Bruch's membrane, extending into the subretinal space and leading to intravitreal hemorrhages. In more advanced cases, patients may present with

symptoms of extraocular extension such as proptosis. In the ocular fundus, the majority of the tumors appear as a pigmented, well circumscribed elevated mass with an associated retinal detachment. Twenty-five percent of the cases are amelanotic (Mukai and Gragoudas 1994). Choroidal melanomas can be classified in two clinical subtypes: nodular and diffuse. Nodular tumors are dome-shaped and may adopt a mushroom shape if the tumor breaks through the Bruch's membrane. The mushroom-shaped growth pattern is almost pathognomonic for uveal melanoma. Diffuse posterior melanomas are less common (4-5% of the cases) (Font, Spaulding et al. 1968), but show a high metastatic risk (24% at 5 years) (Shields, Shields et al. 1996). The 5-year mortality rate for choroidal melanomas was estimated to be 14% (Seddon, Albert et al. 1983).

Uveal melanomas arising from the ciliary body (12% of the cases) (Hungerford 1995) are the most difficult to visualize and therefore, to diagnose. Thus, tumors are often large at the time of diagnosis and most patients present visual alterations. Retinal detachment, hemorrhages, unilateral cataract and secondary glaucoma are commonly observed. Ciliary body melanoma usually presents as a pigmented mass posterior to the pupil. These tumors may also grow in a diffuse fashion, a pattern known as "ring melanoma". Ciliary body melanoma carries the worse prognosis with a 5-year mortality rate of 53% (Seddon, Albert et al. 1983).

Iris melanoma accounts for less than 10% of cases (Egan, Seddon et al. 1988). The patients usually recognize those lesions themselves at an early stage, since they are located in a visible area. Metastasis is uncommon. As a result, iris melanoma carries the

best prognosis with less than 5% mortality at 10 years (Shields, Shields et al. 2001). The management of iris melanoma is generally local resection of the mass.

2.2.4 Diagnosis

Most uveal melanomas are diagnosed by fundus examination (indirect ophthalmoscopy or fundoscopy) either in symptomatic or asymptomatic patients during routine examination. Fundoscopy is the most important diagnostic tool with over 95% of the patients being correctly diagnosed by this method (Mukai and Gragoudas 1994).

Supportive diagnostic methods include ultrasonography, fluorescein and indocyanine green angiography. Ultrasound is particularly useful in cases with opaque ocular media where the posterior pole of the eye cannot be visualized directly. Ultrasound has two modalities: A and B scan. A combination of A-scan and B-scan ultrasonography is usually performed, which presents a 95% accuracy in diagnosing choroidal melanomas (Char 2003). While A-scan shows the reflectivity of the tumor, B-scan provides the shape, location and dimensions of the tumor. Fluorescein and indocyanine green angiography allow for the imaging of the choroidal vasculature. Choroidal melanomas usually have intrinsic tumor vessels, referred to as “second circulation”.

According to the report published by the Collaborative Ocular Melanoma Study Group, the clinical diagnosis of choroidal melanomas has an accuracy of more than 99% when the tumor size is medium or large (COMS 1990). Smaller tumors are harder to diagnose, and usually tumor follow-up is required.

Fine needle aspiration biopsy (FNAB) is usually performed in cases where the diagnosis is uncertain (Shields, Shields et al. 1993). Although this technique carries the risk of visual complications and local spread, it helps in the differential diagnosis of uveal melanoma from simulating lesions and can be safely performed in experts' hands. Recent studies have suggested a new role for FNAB in obtaining cells for cell culture studies particularly because enucleation is now a less frequently used therapeutic method.

2.2.5 Prognostic factors

There are several well-documented prognostic factors of uveal melanoma. They can be classified in clinical and histopathological prognostic factors (Table 1).

Clinical prognostic factors

Tumor size is one of the most important predictors of the patient outcome. The largest tumor dimension (LTD) strongly correlates with prognosis. According to LTD and height, uveal melanomas are classified into small tumors (<10mm LTD and <3mm in height), medium tumors (between 10mm and 15mm LTD and between 3mm and 8mm in height) and large tumors (>15mm LTD and >8mm in height). The 5-year mortality rates are 16%, 32% and 53% respectively (Diener-West, Hawkins et al. 1992).

The location of the tumor is also important, posterior uveal melanomas come with a worse prognosis than anterior uveal melanomas (See section 2.2.3).

<i>CLINICAL</i>	<i>HISTOPATHOLOGICAL</i>
	Cell type
Size	Tumor infiltrating lymphocytes
Location	Vascular loops
	Mitotic activity
	Cytomorphometry
	Extraocular extension

Table 1. Prognostic factors of uveal melanoma

Histopathological prognostic factors

Cell type is a well-known prognostic factor. Two different types of tumor cells can be distinguished: spindle and epithelioid. These two cell types represent different stages of malignancy of the same cell. Spindle cells are fusiform and they are arranged in a fascicular pattern where the cells run parallel to each other. In contrast, epithelioid cells, named after their resemblance with epithelial cells, are large and pleomorphic cells, with large nuclei and nucleoli. Due to their characteristic lack of cohesiveness they can easily infiltrate other tissues and metastasize. According to the Callender classification (Callender 1931) modified by McLean and co-workers (McLean, Foster et al. 1983), tumors can be classified according to the cell type in spindle, epithelioid, and mixed cell tumors. Approximately 56% of the tumors are of mixed cell type (McLean, Foster et al. 1982). Cell type correlates with tumor size. Large tumors are composed of a large number of epithelioid cells, and those tumors carry the worst prognosis.

In contrast with most types of cancers where lymphocytic infiltration is associated with increased survival, for uveal melanoma the presence of tumor infiltrating lymphocytes (TIL) is associated with metastatic disease and thus, poor prognosis (McLean, Foster et al. 1982; Whelchel, Farah et al. 1993). A predominance of T lymphocytes has been observed in most cases. Considering that the eye is an immunoprivileged site, some authors proposed that this phenomenon is likely to be due to a requirement for malignant cell dissemination prior to T cell infiltration (Whelchel, Farah et al. 1993).

Vascular loops, defined by McLean and collaborators as “PAS positive fibrovascular septa that completely surround lobules of tumor cells”, are a sign of poor prognosis (McLean, Keefe et al. 1997). The presence of vascular loops is characteristic of highly aggressive melanomas. Interestingly, it has been shown that these closed loops contain red blood cells but they lack endothelial cells. They consist of channels that are generated by the tumor cells themselves (Maniotis, Folberg et al. 1999). The presence of vascular loops is associated with metastatic death (Mehaffey, Folberg et al. 1997). These channels facilitate tumor perfusion independently of tumor angiogenesis. The ability of melanoma cells to generate microvascular channels is known as vasculogenic mimicry.

Mitotic activity and the mean of the ten largest nucleoli (cytomorphometry) have also been correlated with poor prognosis in patients with uveal melanoma (McLean, Foster et al. 1977; Gamel, McLean et al. 1982; Burnier Pereira, Burnier et al. 2001). They both indicate high cellular activity and proliferation rate. The number of mitotic figures was found to directly correlate with patients' mortality. Cases with high mitotic activity

(9-48 mitotic figures/ HPF) have a higher mortality rate (56% mortality at 6 years) than those with low mitotic activity (0-1 mitotic figures/ HPF), showing a 15% mortality at 6 years (McLean, Foster et al. 1977). The mean of the ten largest nucleoli is closely related to cell type. The former is considered a more objective and reproducible methodology for tumor classification.

The extension of the tumor extraocularly increases the risk of mortality by 2-fold (Seddon, Albert et al. 1983). Extraocular extension was reported to be present in 8% of enucleated eyes (COMS 1998). It is more frequently observed in ciliary body melanomas that are usually larger at the time of diagnosis.

2.2.6 Metastasis

Malignant tumors are characterized by their ability to metastasize. In order to spread from its original site to another part of the body, primary tumor cells have to be able to infiltrate and invade other tissues. Due to the lack of lymphatic channels in the eye, uveal melanoma cells metastasize hematogenously. Although cancers that disseminate in this manner usually develop metastasis in the lung, in the case of uveal melanoma the preferential site of metastasis is the liver (93%) (Albert, Niffenegger et al. 1992; COMS 2001). Moreover, in many cases the liver is the only site of metastasis (30%) as shown on autopsy (Patel, Didolkar et al. 1978). Usually in association with liver, metastases are frequently observed in the lungs (24%), bones (16%) and skin (15%) (Woll, Bedikian et al. 1999; COMS 2001).

Clinically evident metastasis at the time of primary tumor diagnosis is infrequent (1-2% of the cases) (De Croock and Verbraeken 2002). However, almost 50% of the patients will develop fatal metastatic disease within 10 years of diagnosis, regardless of successful treatment of the primary tumor (COMS 2001). The mean survival time following diagnosis of metastasis has been estimated in 6 months (Albert, Niffenegger et al. 1992). This intriguing delayed appearance of metastasis has challenged scientists investigating uveal melanoma for decades. Some authors believe in the presence of CMC and/or occult, dormant, sub clinical micrometastasis at the time of initial intraocular tumor diagnosis (Shields, Shields et al. 1991; McLean 1993; Manschot, Lee et al. 1995).

The early detection of metastasis is critical for the outcome of patients with uveal melanoma. Following diagnosis, patients are screened for the presence of metastasis every six months or once a year. Screening methods include physical examination, liver function tests (LFT) and imaging techniques. Although LFT such as bilirubin and phosphatase alkaline proved to be highly specific, they are associated with poor sensitivity limiting their usefulness (Diener-West, Reynolds et al. 2004). Imaging tests include abdominal ultrasonography and chest X-ray. Again, those methods are highly specific, but they lack sensitivity (Diener-West, Reynolds et al. 2004). Currently, the ability of PET scan to detect occult metastasis is being evaluated (Finger, Kurli et al. 2004). Final diagnosis of metastatic disease is usually accomplished by biopsy.

Even more attractive is the possibility of identifying patients that are at high risk of developing metastatic disease. Based on the assumption that the release of tumor cells from the primary site into the circulation is an early event of the metastatic cascade, the

detection of these cells in the peripheral blood might help in the identification of those patients. The development of sensitive and specific methods for screening of CMC in patients' blood is required. In this sense, RT-PCR has been proposed as a promising tool for the detection of tumor cells in the bloodstream of patients with, among others, breast cancer (Alvero, Burtneß et al. 2004), small cell lung cancer (Yamashita, Matsuo et al. 2002), esophageal cancer (Nakashima, Natsugoe et al. 2003), gastric cancer, colorectal cancer (Huang, Wang et al. 2003), cutaneous melanoma (Stevens, Scheer et al. 1996; Palmieri, Strazzullo et al. 1999; Tsao, Nadiminti et al. 2001; Carrillo, Prados et al. 2002) and even uveal melanoma (Tobal, Sherman et al. 1993). However, further studies are required to determine the usefulness of this molecular serologic tool.

Clearly, a better understanding of the disease may contribute in the design of improved tests for patient screening. The development of methods for the detection of CMC seems to be a promising area of research.

2.2.7 Treatment

During more than a century, enucleation was the only choice of treatment for patients with uveal melanoma. In the late 70's, Zimmerman and co-workers raised the concern whether enucleation could in fact be accelerating the development of metastasis by allowing the dissemination of malignant cells during the surgical procedure (Zimmerman, McLean et al. 1978). This hypothesis motivated researchers to develop alternative therapeutic strategies.

Currently, the management of posterior uveal melanoma depends mainly on tumor size but it is also important to consider patients' age and life-expectancy (Shields, Shields et al. 1996; Woll, Bedikian et al. 1999; Shields 2002; Char 2003). Small tumors are usually observed every three months to determine tumor growth. For medium size tumors, the most widely used alternative treatment is radiation therapy. Different modalities of radiotherapy including proton beam therapy and plaque brachytherapy are employed, the latter being the most popular option. Other conservative treatment options include transpupillary thermotherapy, laser photocoagulation and cryotherapy. Enucleation with low manipulation of the affected eye with or without preoperative radiotherapy is still considered for large advanced tumors and for those cases with optic nerve involvement. However, no therapeutic benefits were demonstrated with preoperative radiotherapy. Orbital exenteration is usually performed in cases with massive extraocular extension.

Despite the adoption of alternative local treatments, survival rates for patients with uveal melanoma have remained unchanged (Singh and Topham 2003) and thus, the management of posterior uveal melanoma is still highly controversial. The peak of mortality observed by Zimmerman and co-workers, 2-3 years after enucleation, has also been observed with the use of alternative therapies (Singh, Rennie et al. 2004). These findings support the idea of early dissemination and the presence of micrometastasis at the time of initial diagnosis (De Croock and Verbraeken 2002).

Little progress has been made in the treatment of metastatic uveal melanoma. The available treatment options are palliative surgery for patients with solitary hepatic metastasis, chemoembolization with cisplatin or intra-arterial chemotherapy with carboplatin or fotemustine for patients with metastatic disease limited to the liver, and systemic therapy using BOLD (bleomycin, vincristine, lomustine and dacarbazine) regimen in combination with interferon for patients with extrahepatic disease (Woll, Bedikian et al. 1999; Shields 2002). However, to date no therapeutic modality has proven to prevent, efficiently treat or significantly extend the survival of patients with metastatic disease. The efficacy of immunotherapy using either interferon or IL-2 usually in combination with chemotherapy continues to be evaluated. Clinical trials are being conducted to elucidate the usefulness of adjuvant therapies at the time of primary tumor treatment. In the future, novel therapeutic options to prevent the development of metastasis or to combat established metastasis may arise from research participation.

2.3 Animal models of disease

2.3.1 Animal models of cancer

Most of the medical knowledge gained in the last century has involved work with laboratory animals. In cancer research, animal models help us better understand disease progression and spread, processes that can occur only *in vivo*. By definition, an animal model of a particular disease must be reproducible and predictable. The most popular animal models of disease are experimental (also known as induced) and spontaneous, the former being the most widely used.

2.3.2 Uveal melanoma animal models

During the last several decades, a major challenge facing researchers investigating uveal melanoma has been to develop a suitable animal model for the study of this disease. Ideally, a good animal model of uveal melanoma should share features with the human disease on various levels. The rate of development of intraocular tumors should be high, and tumors should display similarities in morphology, molecular biology and behavioral characteristics to human uveal melanoma. Metastatic disease should be present in a high proportion of animals. In addition, the disease should progress over a short period.

Both spontaneous and experimental animal models of uveal melanoma have been described in the literature. However, the spontaneous occurrence of uveal melanoma in dogs (Diters, Dubielzig et al. 1983; Ryan and Diters 1984) and cats (Acland, McLean et al. 1980) has limited utility as a model since the disease occurs rarely in these animals and its biologic behavior differs from that of the disease in human beings. In experimental models of uveal melanoma the disease can be induced in the host animal by inducing transformation of normal melanocytes into malignant melanoma cells using oncogenic agents, or by directly inoculating cultured melanoma cells or xenografts into the eyes of the host animal.

2.3.2.1 Inducing transformation of melanocytes

The main advantage of these models is that they provide a tool for the study of the pathogenic process of malignant transformation in normal melanocytes. However, studies have not always achieved the specific transformation of uveal cells *in vivo*. Other cell types may suffer the influence of the mutagenic agent used, resulting in the simultaneous

development of several cancers. A better approach may be to transform uveal cells *in vitro* for subsequent inoculation. In addition, metastasis in this model was either of unclear origin or not present at all in most cases.

Oncogenic viruses

Oncoviruses, including both RNA and DNA viruses, were evaluated for their potential to induce malignant transformation of the uvea. Albert and colleagues (Albert, Shadduck et al. 1981) induced uveal melanoma in kittens by injecting the feline leukemia-sarcoma virus (FeLV-FeSV) into the anterior chamber of the animals. Attempts to separate FeLV from FeSV were unsuccessful. Intraocular tumors that were clinically detectable 40 days after virus injection developed in the iris and ciliary body in a high proportion of animals (89%). Disadvantages of this model include the high death rate of the animals owing to disseminated viral infections, the presence of viral particles in the tumor cells and the inability to determine whether extraocular neoplasms were a result of the viral infection or true metastatic disease. The simian virus 40, a DNA oncovirus, was used to induce neoplastic transformation of hamster uveal cells *in vitro* (Albert, Rabson et al. 1968). Subcutaneous injection of these cells into hamsters resulted in the development of a variety of tumors that gradually dedifferentiated, losing their pigmentation and lacking premelanosomes.

Carcinogenic substances

A variety of carcinogenic substances have been used to induce uveal malignant proliferation in laboratory animals. Ethionine, *N*-2-fluorenylacetamide and methylcholanthrene were tested in rodents, resulting in the development of intraocular

tumors that varied in morphology and time of development (Patz, Wulff et al. 1959; Benson 1962). No metastasis was detected. Chemical induction was also achieved with nickel subsulfide in rats (Albert, Shaddock et al. 1980). Intraocular tumors developed 6 to 9 months later, and widespread metastasis was observed. The use of 7,12-dimethylbenz[a]anthracene (DMBA) for the induction of primary uveal melanocytic lesions in rabbits has also been reported (Pe'er, Folberg et al. 1992). After a 16-week treatment with DMBA and croton oil, treatment was stopped and tumor regression was observed.

Transgenic mice

Transgenic mouse models use the tyrosinase promoter to target the expression of oncogenes, such as the simian virus 40 early region transforming sequences (Bradl, Klein-Szanto et al. 1991; Anand, Ma et al. 1994) and the mutated human T24 Ha-ras gene (Kramer, Powell et al. 1998), in melanin-producing cells. In one study most of the pigmented ocular tumors, arising from both uveal melanocytes and retinal pigment epithelium, were benign (Kramer, Powell et al. 1998). In addition, melanocytic hyperplasia was found in the skin, inner ear and meningeal membranes in the brain. No metastasis was demonstrated.

2.3.2.2 Direct intraocular inoculation of melanoma cells

Injections of melanoma cell suspensions or transplantations of tumor xenografts into a host animal eye have led to the successful development of malignant intraocular tumors and metastasis in some cases. Although this approach does not allow for the study of the malignant transformation process, it often provides more reproducible and predictable models that better resemble the disease in human beings. It also avoids the

need to work with oncogenic agents. Cell suspension inoculations have been used in most studies. Xenotransplantation may be limited by the fact that implanted fragments may contain areas of tumor necrosis, which reduce the viability of the tumor fragment. Consequently, the rate of tumor development is lower (Bonicel, Michelot et al. 2000).

The site of melanoma cell implantation may influence tumor behavior and site of metastasis. Therefore, orthotopic models may be superior to heterotopic models. Morphologic cell changes after subcutaneous transplantation of human choroidal melanoma xenografts in nude mice were attributed in part to heterotopic transplantation (Heegaard, Spang-Thomsen et al. 2003). Using an orthotopic model for uveal melanoma in severe combined immunodeficient mice, Mueller and associates (Mueller, Maniotis et al. 2002) highlighted the advantages of using their model for the study of microcirculation patterns of uveal melanoma. Whereas subcutaneous injection of human uveal melanoma cells into immunosuppressed mice resulted in tumors with necrosis and angiogenesis, usually uncommon features of primary human uveal melanomas, orthotopic injection of these cells in rabbits resulted in tumors that reproduced the internal architecture and microcirculation patterns of human uveal melanomas (Mueller, Folberg et al. 1999). In the development of animal models of uveal melanoma three different host animals (hamster, mouse and rabbit) and cells lines derived from three different donors (hamster, mouse and human) were used. Almost every possible combination has been reported in the literature.

Hamster Greene melanoma

The most widely used cell line in experimental models of uveal melanoma to date is the hamster Greene melanoma. This cell line was isolated by Greene (Greene 1958) in 1958 from a skin melanoma that spontaneously occurred in a hamster. Following orthotopic transplantation into its natural host, the Greene melanoma produced highly aggressive tumors characterized by necrosis, haemorrhages and extraocular extension that filled the eye in 3 to 4 weeks. Although widespread metastasis was suspected, its origin was unclear owing to the presence of an endogenous retrovirus associated with the cell line that was able to transform adjacent tissues and cause distant infections (Albert, Lahav et al. 1974). The amelanotic cell line derived from this hamster melanoma showed increased metastatic potential and acquired the ability to be implanted in heterologous hosts (Greene and Harvey 1966). To overcome the practical problems associated with the small size of the hamster eye, this cell line was used to establish a rabbit model (Greene and Harvey 1966; Krohn, Brandt et al. 1970). The latter model was used extensively to evaluate therapeutic strategies, including photodynamic therapy (Ozler, Nelson et al. 1992; Schmidt-Erfurth, Flotte et al. 1996), chemotherapy (Skov, Kumi et al. 1987; Olsen, Blumenkranz et al. 1988) and radiotherapy (Fournier, Saulenas et al. 1985; Yang, Olsen et al. 1993). A major limitation associated with the use of the hamster Greene melanoma model to evaluate therapies is the different biologic behavior of a cutaneous hamster melanoma compared to the human counterpart (Dithmar, Albert et al. 2000). In addition, early necrosis and tumor shrinkage, not overcome by immunosuppressive treatment (de Waard-Siebinga, van Delft et al. 1994), limit the time in which therapeutic benefits can be demonstrated (Romer, van Delft et al. 1992).

B16 mouse melanoma

In 1954 the B16 melanoma cell line was isolated from a spontaneously occurring cutaneous murine melanoma (Green 1962). For the most part, B16 cell lines were used as a model of metastatic melanoma. Several cell lines with different metastatic potential, including B16-F10, B16-F10 Queens and B16-LS9, were derived from the original culture. Most of the studies have been carried out in mice in which no immunosuppression is required. Therefore, this model allows for the study of immune system influence on the metastatic process.

There has been a great deal of research to elucidate the relation between natural killer cell activity and the metastatic behavior of this cell line. However, the role of natural killer cells in melanoma progression remains unclear (Nieder Korn 1984; Yokoyama, Yoshie et al. 1989). The rate of metastasis following injection of these cell lines was found to be dependent on the inoculation site (anterior compartment or posterior compartment) but not on the concentration of the inoculum (Harning, Koo et al. 1989; Grossniklaus, Barron et al. 1995). B16 melanoma usually metastasizes to the lung (Grossniklaus, Barron et al. 1995; Grossniklaus, Wilson et al. 1996). However, Rusciano and coworkers (Rusciano, Lorenzoni et al. 1994) reported the ability of B16-LS9 cells to also metastasize to the liver in mice. In a study evaluating the differences in metastatic behavior between melanomas arising in the anterior compartment and those arising in the posterior compartment, Grossniklaus and colleagues (Grossniklaus, Barron et al. 1995) mentioned the technical difficulties of accurately injecting the cell inoculum into the posterior compartment of the mouse eye. In this sense, the larger size of the rabbit eye provides a more suitable model for the study of choroidal melanomas: not only is cell

injection into the posterior compartment more precise, but, also, conventional diagnostic techniques can be used for intraocular tumor examination. B16 cells were successfully transplanted into the posterior compartment of rabbits (Hu, Huh et al. 1994; Pineda, Theodossiadis et al. 1998). The establishment of rabbit models of pigmented choroidal melanomas with pulmonary metastasis (Hu, Huh et al. 1994) and extraocular extension (Pineda, Theodossiadis et al. 1998) have been reported. However, successful intraocular tumor growth in this heterologous host requires immunosuppression, and this cell line still shows the limitation of not being of human origin.

Human uveal melanoma cell lines

Although the early models of uveal melanoma used cutaneous melanoma cells from animals, during the past few years more attention has been paid to the choice of cell type used. A major advance in uveal melanoma research has come from the use of human uveal melanoma cell lines to develop improved experimental models. Initial experiments used fresh cells from patients with uveal melanoma to induce intraocular tumor development in athymic nude mice (Albert, Wagoner et al. 1980). This model provided a readily accessible source of human uveal melanoma cells maintained *in vivo* through serial transplantation for extensive characterization.

Today, a large number of established primary and metastatic uveal melanoma cell lines, such as the OCM (Albert, Wagoner et al. 1980), MKT-BR (Diebold, Blanco et al. 1997), SP6.5 (Diebold, Blanco et al. 1997), 92.1 (De Waard-Siebinga, Blom et al. 1995) and OMM (Luyten, Naus et al. 1996), are available for research. The orthotopic inoculation of human uveal melanoma in rabbits immunosuppressed with cyclosporin A

(CsA) was first described by Kan-Mitchell and associates (Kan-Mitchell, Mitchell et al. 1989), in 1989. This model is still the most powerful tool for the study of uveal melanoma since it combines the advantages of using both human uveal melanoma cells and rabbits. The disadvantage of this approach compared with the athymic nude mice model is the need for daily administration of CsA, a drug that can cause severe side effects in animals and therefore limit the length of the experiment. Recent research has focused on establishing whether the use of CsA is strictly required until the end of the experiment to maintain tumor growth (Ordóñez, Saornil et al. 2002). Studies have also determined the appropriate dosages of CsA that result in minimum side effects, leading to longer survival and further progression of the disease. Ordoñez and coworkers (Ordóñez, Saornil et al. 2002) concluded that continuous immunosuppression is needed to avoid tumor rejection. They observed signs of tumor regression 3 weeks after stopping the administration of CsA. Based on their studies comparing different dosages of CsA, Blanco and colleagues (Blanco, Saornil et al. 2000) proposed a convenient dosage schedule that improves survival rates and produces a high percentage of intraocular tumors, with development of metastatic disease in 26.8% of the animals.

Chapter 3

THESIS PURPOSE AND OBJECTIVES

The purpose of this thesis is to characterize the processes of tumor development, malignant cell dissemination and metastasis in a 10-week animal model of uveal melanoma.

This work has the following objectives:

- 1) To document the progression of uveal melanoma in the albino rabbit model
- 2) To determine intraocular tumor development rate using different diagnostic tools
- 3) To characterize the intraocular tumors and evaluate prognostic factors of uveal melanoma
- 4) To evaluate the presence of viable CMC in the peripheral blood of these animals
- 5) To test the potential of RT-PCR for CMC detection in rabbits' blood
- 6) To determine the rate, time course, and site of metastasis in this animal model

Chapter 4

MATERIALS AND METHODS

The animal model was carried out in compliance with the Association of Research in Vision and Ophthalmology Resolution on the use of Animals in Ophthalmic and Visual Research. The approval of both, the Animal Care Committee and the Ethics Subcommittee at McGill University, was obtained prior to all experiments (Appendix).

4.1 Animals

Twenty-seven male New Zealand albino rabbits (Charles River Canada, St-Constant, Quebec, Canada) with a mean initial weight of 3.3 ± 0.2 kg (range: 2.9-3.6 kg) were used in this study. The animals were allowed to acclimatize for 1 week before the experiments. They were kept in individual steel cages in the animal facility at the Lyman Duff building (Figure 1).

The number of animals desired for the experiment was 25. Three additional animals were required in case of surgical failure. No surgical failure occurred, therefore two of them were included in the experiment and the remaining rabbit was used as negative control. The decision to use only male rabbits was based on the fact that females undergo hormonal changes that may interfere with the experiment. Albino rabbits have been chosen because the ocular examination in these animals is more accurate than that in pigmented rabbits. Animals with an initial weight of 3 kg or greater were chosen to better resist the toxic effects of CsA.

4.2 Immunosuppression with Cyclosporin A

The animals were immunosuppressed using CsA (Sandimmune 50 mg/ml; Novartis Pharmaceuticals Canada Inc., Dorval, Quebec, Canada) in order to prevent cell

rejection. The animals received daily intramuscular injections of CsA in the thigh muscles, alternating right and left side every day, using a syringe (Becton Dickinson, New Jersey, USA) with a needle size of 23G 1 (Becton Dickinson). CsA administration was maintained throughout the 10-week experiment to prevent spontaneous tumor regression. The dosage schedule recommended in previous studies (Blanco, Saornil et al. 2000) was employed: 15mg/kg/day 3 days before cell inoculation and during 4 weeks thereafter, followed by 10mg/kg/day during the last 6 weeks of the experiment. CsA doses were adjusted weekly according to the animal weight.

4.3 Cell line and cell culture

The 92.1 human uveal melanoma cell line (De Waard-Siebinga, Blom et al. 1995) was used. This selection was based on previous studies performed in our laboratory where these cells showed a higher *in vitro* proliferation and/or invasion rate than other human uveal melanoma cell lines (UW-1, OCM-1, MKT-BR). The cells were maintained at 37°C in a humidified 5% CO₂-enriched atmosphere (Thermo Forma Series II Water Jacketed CO₂ Incubator, Fisher Scientific Limited, Ontario, Canada). The cells were grown in 25 cm² cell culture flasks (Fisher Scientific Limited) in RPMI-1640 media (Invitrogen, Burlington, Ontario, Canada); supplemented with 5% heat inactivated fetal bovine serum (FBS; Invitrogen), 1% fungizone (Invitrogen), and 1% penicillin-streptomycin (Invitrogen). Cell count and viability were estimated by trypan blue exclusion technique. The cells were routinely observed with an inverted phase contrast microscope to evaluate cell density and to rule out the possibility of contamination. Once monolayer cultures were confluent, cells were passaged using 0.05% trypsin in EDTA (Fisher Scientific Limited).

4.4 Cell injection procedure

The surgical procedure was carried out under aseptic conditions (Figure 2). The right eye of the rabbit was dilated 10 minutes before operation using drops of phenylephrine hydrochloride (Mydfrin, Alcon Canada Inc., Mississauga, Canada) and tropicamide (Mydriacyl, Alcon Canada Inc.) for ophthalmoscopic examination. A fundoscopic examination, using an indirect binocular ophthalmoscope, was performed on each rabbit to rule out any ocular pathology. Ocular anesthetic (Proparacaine Hydrochloride; Alcaine, Alcon Canada Inc.) was applied topically in the right eye of the animal one-minute before the surgery. Ketamine (35mg/kg; Vetalar, Vetrepharm Canada Inc., Belleville, Ontario, Canada) and xylazine (5mg/kg; Anased, Novopharm Limited, Toronto, Ontario, Canada) were used as general anesthetics during the surgical procedure.

The injection of the cells was performed according to a previously described technique (Blanco, Saornil et al. 2000). Briefly, an incision of 2-3 mm was made in the temporal superior sclera (sclerotomy) up to the suprachoroidal space, which is recognized by a brown color, using an ophthalmic surgical blade. If a vitrectomy (perforation of the vitreous cavity) had occurred, the animal would not have been used for the experiment. A paracentesis of the anterior chamber of the eye (removal of aqueous humor) was performed using an insulin-like syringe (Becton Dickinson) to release intraocular pressure. The extracted volume was ~ 0.2 ml. A tunelizer was used to make a tunnel in the suprachoroidal space by the incision already made. One end of the incision was sewed forming a U. An insulin-like syringe with the canulla loaded with one million cells (cellular viability greater than 98%) suspended in 0.1 ml of RPMI-1640 media was

introduced into the tunnel and the cells were injected. The suture was knotted when the canulla was extracted.

Post-operative fundoscopy was performed to discard immediate complications. Antibiotic ointment (BNP, Vetcom Inc., Upton, Quebec, Canada) was then applied to the eye. In the first two days after surgery, buprenorphine (0.02 mg/Kg; Temgesic, Schering-Plough, Pointe Claire, Quebec, Canada) was administered when determined necessary by the animal health technician and veterinarian.

Figure 1. Lyman Duff building animal care facility

Figure 2. Cell injection surgical procedure

4.5 Follow-up

4.5.1 Ophthalmologic examination

Indirect ophthalmoscopy of dilated pupils (Mydrin, Alcon Canada Inc.; Mydrin, Alcon Canada Inc.) was performed before cell inoculation, to rule out any ocular pathology, and weekly after cell inoculation to clinically document intraocular tumor development. Photographs of the findings were captured by a digital camera (Kodak DCS 620, Kodak Canada Inc., Toronto, Ontario, Canada) mounted on the fundus camera (Zeiss FF450 plus, Carl Zeiss Canada Ltd., Ontario, Canada). Tumors were classified in different stages according to the fundoscopic observations to evaluate tumor growth. Table 2 shows the criteria for intraocular tumor classification.

<i>STAGE</i>	<i>OBSERVATION</i>
0	No findings
1	Localized lesion
2	Tumor involving one quadrant of the retina
3	Tumor involving two quadrants of the retina
4	Tumor involving two or three quadrants of the retina
5	Tumor with extraocular extension

Table 2. Intraocular tumor classification criteria by fundoscopy

(Modified from Blanco 1996)

Ultrasound examination using a 10 MHz B-scan and standardized A-scan (I³ System-ABD™, Innovative Imaging Inc., Sacramento, California, USA) was performed

in 15 animals under anaesthesia during the last week of the experiment to determine the echographic characteristics, location and final dimensions of the tumors *in vivo*.

4.5.2 Cyclosporin A toxicity

A study of CsA toxicity throughout the 10-week experiment was performed. The animals were daily monitored for signs of CsA toxicity including regurgitation, diarrhea and weight loss. In addition, once a week the animals' general appearance was evaluated. Each observation was given a score as shown in Table 3. The sum of all the animals' scores per week was divided by the total number of animals in the corresponding week, which results in the mean score value for the week. The value of this score could range from 0 to 6 in the worst-case scenario.

<i>OBSERVATION</i>	<i>SCORE</i>
Regurgitation	1
Diarrhea	1
Weight loss	1
Appearance	
Good	0
Regular	1
Bad	2
Pre-mortem	3

Table 3. Cyclosporin A toxicity evaluation

(Modified from Blanco 1996)

4.6 Euthanasia

In order to document the time-course of the disease, particularly of metastasis development, 1 animal was euthanized per week. The selection criterion was based on the animal appearance and signs of CsA toxicity. The remaining rabbits were sacrificed at the end of the experiment. In addition, animals presenting signs of pain or distress any time during the experiment were immediately sacrificed, as indicated by the veterinarian.

The method of euthanasia was exsanguination following anesthesia using intramuscular ketamine-xylazine (35 mg/kg-5 mg/kg). An autopsy was performed on every animal that had been sacrificed. The enucleated eyes and the organs with possible metastatic disease such as lungs, livers and kidneys from each animal were collected. The specimens were macroscopically examined, recording any pathological observation, and preserved in 10% phosphate buffered formalin.

4.7 Post-mortem studies

4.7.1 Histopathological studies

Formalin-fixed, paraffin-embedded sections of the collected specimens including eyes, lungs, livers and kidneys, were Hematoxylin and Eosin (H&E) stained for histopathologic assessment. The enucleated eyes were microscopically examined to determine tumor presence and morphology. A study of intraocular tumor progression was performed throughout the 10-week experiment. The lungs, livers and kidneys were inspected for the presence and form of presentation of metastatic disease. Other general histopathological findings were also recorded.

Uveal melanoma histopathological prognostic factors were evaluated in the intraocular tumors, weekly during the course of the experiment and in 10 animals at the end of the experiment. The prognostic factors evaluated included cell type, tumor size, tumor infiltrating lymphocytes (TIL), vascular loops, mitotic rate, cytomorphometry (Mean of the Ten Largest Nucleoli) and extraocular extension. Tumors were classified into either the “high lymphocytic” group or “low lymphocytic” group according to the number of TIL (100 or more and less than 100, respectively) present per 20 high-power (X400) fields (HPF) as previously described (de la Cruz, Specht et al. 1990). Eye sections were stained with periodic acid Schiff (PAS) to evaluate the presence of vascular loops (McLean, Keefe et al. 1997). Mitotic activity was measured as the mean number of mitoses seen per 10 HPF. The Mean of the Ten Largest Nucleoli (MTLNI) in 25 HPF was measured (Burnier Pereira, Burnier et al. 2001).

4.7.2 Immunohistochemistry

Immunohistochemistry using the HMB-45 monoclonal antibody (mAb) (DakoCytomation, Mississauga, Ontario, Canada) was performed according to the streptavidin-biotin complex method. Tissue sections of 4 μ m thick were deparaffinized with xylene and rehydrated in graded ethanol series. The sections were washed for 10 minutes with 3% hydrogen peroxide and methanol to block the activity of the endogenous peroxidase. Microwave citrate treatment (citrate buffer pH 6.0) was used as the heat antigen retrieval technique. In order to block non-specific binding of the antibodies, the slides were washed for 30 minutes in 1% bovine serum albumin (BSA) and tris buffered saline (TBS; pH 7.6). The sections were incubated for 1 hour at 37°C with the mouse

mAb against HMB-45 (dilution 1:50) in a humidity chamber. On negative control sections, non-immune serum (0.1% BSA/TBS) was applied instead of the primary antibody. The sections were then incubated with the biotinylated rabbit anti-mouse secondary antibody (dilution 1:500; DakoCytomation) for 30 minutes at 37°C. Subsequently, horseradish peroxidase conjugated streptavidin-biotin complex (DakoCytomation) was applied to the sections and they were incubated for 30 minutes at 37°C. The visualization of the antibody-antigen reaction was achieved by using 3-amino-9-ethylcarbazole (AEC) chromogen (DakoCytomation). The sections were then counterstained with hematoxylin, mounted with an aqueous mounting medium (Faramount, DakoCytomation) and coverslipped.

4.7.3 Culture of intraocular tumor and metastatic cells

Following autopsy of the sacrificed animals, samples were immediately taken from the intraocular tumor and any macroscopically observed metastasis. Collected samples were homogenized using 0.05% trypsin in EDTA (Fisher Scientific Limited) at 37° C for 10 minutes, washed and re-suspended in fresh culture media. Cells were then seeded into 6 well plates (Becton Dickinson) and placed in a 5% supplemented CO₂, 37°C incubator. After three days of incubation the media was changed. Cells were grown to confluence and subcultured. A portion of each culture was preserved frozen in liquid nitrogen.

Cell cultures were used for cytopins or grown on coverslips for immunostaining. Briefly, cells were washed, re-suspended in 1 ml PBS and 2×10^5 cells in 0.3 ml were spun

onto glass slides at 30 xg for 3 minutes using a cytopsin centrifuge (Shandon Scientific Limited, Runcorn, England). Cytospins were fixed in 2% paraformaldehyde for 20 minutes at room temperature (RT). Additionally, to better preserve cellular morphology, cells were grown on coverslips. Cultures usually reached 80-90% confluence after 2-3 days. Coverslips were then washed twice with PBS and fixed with 100% methanol for 10-20 minutes at 4°C. Uveal melanoma cells were identified through specific immunostaining using HMB-45, Melan A (dilution 1:25; Novocastra Laboratories Ltd, Burlington, Ontario, Canada) and Tyrosinase (dilution 1:25; Novocastra Laboratories Ltd.) mouse mAbs. 92.1 cells served as a positive control. The immunostaining protocol used was similar to that described in section 4.7.2.

4.8 Blood sample processing

4.8.1 Blood collection

Two techniques for blood collection were employed in these studies: central ear artery extraction and cardiac puncture. Central ear artery blood was used to spike human uveal melanoma cells for PCR detection. Blood samples obtained by cardiac puncture were used to isolate CMC for *in vitro* studies.

A total of ~ 25 ml of blood was collected (~ 2 ml extracted from each rabbit) prior to the beginning of the project. The animals were sedated using intramuscular injections of hypnorm (fentanyl citrate 0.315mg/ml and fluanisone 10mg/ml; Janssen Pharmaceutica, Beerse, Belgium) at a dose of 0.3 ml/kg. The blood samples were extracted from the central ear artery, which was vasodilated by gentle friction.

Subsequently, the hub of a 20G1½ needle (Becton Dickinson) was broken off and the shaft was inserted into the vessel. The blood dripped from the needle into the 6 ml blood collection tube containing K₂ EDTA 10.8mg (Becton Dickinson) (Figure 3).

The animals were anesthetized using intramuscular ketamine-xylazine (35 mg/kg-5 mg/kg) prior to cardiac puncture at the moment of euthanasia. The animals were placed in dorsal recumbency and a 20G1½ blood collection needle (Becton Dickinson), attached to a blood collection tube holder (Becton Dickinson), was inserted where the ribs meet the xiphoid towards the palpable beat. Blood samples were collected into 6 ml blood collection tubes containing K₂ EDTA 10.8mg (Becton Dickinson).

4.8.2 Culture of circulating malignant cells

The presence of CMC was assessed by culturing the mononuclear layer isolated from whole blood samples obtained at the moment of euthanasia, starting 6 weeks after cell inoculation. Mononuclear layers from 19 rabbits were isolated and then cultured.

The mononuclear cells were isolated utilizing the Ficoll-Paque Plus method (Amersham Biosciences, Baie d'Urfé, Quebec, Canada). Ficoll-Paque Plus is a density-gradient procedure used to separate the different blood cell types into layers (Figure 4). Two ml of fresh rabbit blood was diluted in 2 ml of PBS and mixed. The diluted blood sample (4 ml) was carefully layered on top of a 15 ml centrifuge tube containing 3 ml of Ficoll-Paque solution and centrifuged at 400 xg for 30-35 minutes at 18-22°C. The upper layer of plasma was removed using a Pasteur pipette and stored at -20°C for future studies. The mononuclear layer containing the malignant cells was transferred, using a

Pasteur pipette, to a sterile tube and centrifuged at 120xg for 10 minutes. The supernatant was removed and the resulting pellet was re-suspended in 2 ml of 10% FBS supplemented RPMI-1640 media. Cells were then seeded into a 6 well plate (Becton Dickinson) and placed in 5% supplemented CO₂, 37°C incubator. After three days of incubation the media was changed and all non-adherent cells were washed off. Cells were grown to confluence and subcultured. Each culture was preserved frozen in liquid nitrogen for future studies.

Cells were either grown on coverslips or deposited onto coverslips for immunostaining with HMB-45, Melan-A and Tyrosinase using 92.1 cells as positive control, similarly as previously described (See section 4.7.3).

4.8.3 Spiking of human uveal melanoma cells into rabbits' blood

The potential of PCR technique to detect CMC in rabbit peripheral blood samples was evaluated by spiking different densities of 92.1 human uveal melanoma cells into rabbits' blood.

A 92.1 cell stock containing 1×10^6 cells/ml was used to prepare serial dilutions 1/10 in PBS obtaining cell suspensions containing: 1×10^5 , 1×10^4 , 1×10^3 , 1×10^2 cells/ml. Each sample, including the stock, was further diluted 1/10 in PBS obtaining cell suspensions containing: 1×10^5 , 1×10^4 , 1×10^3 , 1×10^2 and 10 cells/ml. This procedure was performed in duplicate. Two ml of each suspension, including the undiluted cell stock containing 1×10^6 cell/ml, were diluted in 2 ml of whole rabbit blood (extracted prior the

experiments) obtaining the 4 ml diluted blood sample required for Ficoll-Paque method. As a result, 6 cell densities were tested: 1×10^6 , 1×10^5 , 1×10^4 , 1×10^3 , 1×10^2 and 10 cells/ml. Negative control samples consisted of a mixture of 2 ml of fresh rabbit blood and 2 ml of PBS containing no cells. The Ficoll-Paque separation procedure continues as previously described. Once the mononuclear layer was isolated, it was transferred into a clean centrifuge tube and 10 ml of PBS were added and the cell suspension homogenized. The sample was centrifuged at 100xg during 10 minutes at 18-22°C. The supernatant was removed and the resulting pellet was stored at -80°C until RNA extraction.

4.8.4 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

The detection of the spiked cells was performed by amplification of the Tyrosinase and Melan A messenger RNA by RT-PCR and nested RT-PCR. Total cellular RNA was isolated from the pellets obtained by Ficoll-Paque Plus method, using TRIzol reagent (Gibco BRL, Burlington, Ontario, Canada) according to manufacturer's instructions. RNA concentration and purity were determined spectrophotometrically at 260 and 280 nm using an Ultrospec 2000 spectrophotometer (Biochrom Ltd., Cambridge, UK). RNA samples (1 µg) were further purified from residual DNA contaminants by digestion with DNase I (Deoxyribonuclease I, Amplification Grade; Invitrogen) for 15 minutes at RT prior to reverse transcription. The enzyme was inactivated by addition of EDTA and heated for 10 minutes at 65°C.

The first strand cDNA was obtained by reverse transcription using the Moloney Murine Leukemia Virus-Reverse Transcriptase (MMLV-RT; Invitrogen) at 37°C for 1 hour according to the product protocol. The 20 µl reaction mixture consisted of the

DNase digested product (11 μ l), dNTP mix 10mM [1 μ l, 100 μ M of each dNTP (dATP, dGTP, dCTP and dTTP); Invitrogen], DTT (1 μ l, 0.1 M; Invitrogen), random hexamers (1 μ l, 5 A260 U; Amersham Biosciences), RNA guard (1 μ l, 39,939 U/ml; Amersham Biosciences), 5 x first strand buffer (4 μ l; Invitrogen) and MMLV-RT (1 μ l, 200U/ μ l). The reaction was inactivated by heating at 95°C for 10 minutes. The reaction in which the MMLV-RT was omitted was used as reverse transcription negative control. Amplification of the cDNA sequences of interest was performed in a PTC-100 Programmable Thermal Controller (MJ Research Inc., Massachusetts, USA) using the Platinum Taq DNA Polymerase (Invitrogen). The reaction mixture (50 μ l) was prepared for PCR including the cDNA template (5 μ l), dNTP mix 10 mM (0.5 μ l, 100 μ M of each dNTP; Invitrogen), MgCl₂ final concentration 1.5 mM (3 μ l, 25 mM), the primers (sense: 5' CACTCTTACACCACGGCTGA & antisense: 5' TAAGGTGGTGGTGACTGTTCTG for Melan A and sense: 5' TTGGCAGATTGTCTGTAGCC & antisense: 5' AGGCATTGTGCATGCTGCTT for Tyrosinase; Sheldon Biotechnology Centre, McGill University) (1 μ l, 0.5 mM), 10x PCR buffer (5 μ l; Invitrogen) and Taq DNA Polymerase (0.2 μ l, 5U/ μ l) in Milli-Q H₂O. Reaction in which the cDNA was omitted was used as PCR negative control. The PCR cycling protocol consisted of 30 cycles of denaturation at 94°C for 4 minutes, annealing at 55°C for 1 minute, and extension at 72°C for 10 minutes, followed by final extension at 72°C for 5 minutes. The amplification products (284 bp and 264 bp for Tyrosinase and Melan A, respectively) were analyzed by agarose gel (1%; Invitrogen) electrophoresis and visualized by ethidium bromide staining.

To improve the method sensitivity and specificity a second amplification round using internal primers (sense: 5' TCATCGGCTGTTGGTATTGTAG & antisense: 5'

ATAAGCAGGTGGAGCATTGG for Melan A and sense: 5' GTCTTTATGCAATGGAACGC & antisense: 5' GCTATCCCAGTAAGTGGACT for Tyrosinase; Sheldon Biotechnology Centre, McGill University) was performed. The reaction mixture (50 µl) for nested PCR was basically the same as for PCR, but instead of cDNA, the first amplification product was re-amplified (1 µl). This time negative controls were prepared omitting the addition of the PCR product. This technique, nested RT-PCR, renders two new amplification products (207 bp and 245 bp for Tyrosinase and Melan A, respectively), which were obtained and analyzed similarly to those of the first amplification round.

Figure 3. Blood extraction from the rabbits' central ear artery

Figure 4. Isolation of mononuclear cells from whole blood samples

Blood samples were separated in layers containing different cell types using Ficoll-Plaque Plus: 1) Plasma; 2) Mononuclear cells; 3) Ficoll-Paque; 4) Granulocytes; 5) Erythrocytes.

Chapter 5

RESULTS

5.1 Survival rate and animal general condition

The 10-week animal model was carried out without major complications. The survival rate in this model was 90%. Since one animal was sacrificed per week, 19 of the initial 27 animals were supposed to survive until the last week of the model. Seventeen rabbits survived until the end of the experiment. Only 2 rabbits had to be euthanized before planned, the first one during the first week and the other in week 8, due to poor general conditions.

The animals remained in good condition throughout the experiment, presenting only minor signs of CsA toxicity including occasional diarrhea or, in some cases, regurgitation. We observed an initial acute weight loss 1 week after cell injection that can be partly attributed to the surgery. After week 1, the animals maintained their weight until the end of the experiment (Figure 5).

The results of the evaluation of the other signs of CsA toxicity are presented in Figure 6. One week after cell injection the mean score increased mainly due to weight loss. Only after week 5 the mean score slightly increases again. However, it was never more than 1.1, which represents a considerable low value taking into account that CsA toxicity was pointed out as one of the major limitations of this model.

Note that in the figures presented in this work the week number corresponds to the number of weeks after cell inoculation.

Figure 5. Animal weight during the experiment

The mean animal weight was calculated for the number of remaining animals each week. Values presented are the mean $\pm$ standard deviation.

Figure 6. Cyclosporin A toxicity evaluation during the experiment

The mean toxicity score was calculated for the number of remaining animals each week. Values presented were calculated as the sum of all animals' scores per week divided by the total number of animals in the corresponding week.

5.2 Intraocular tumor studies

5.2.1 Monitoring intraocular tumors *in vivo*

According to the fundoscopic examination performed before the cell injection procedure, the animals were free of ocular disease at the beginning of the experiment. During the 10-week experiment, fundoscopy allowed for the detection of intraocular tumors in 67% of the animals (n=27) (Figure 7). Some tumors (7 out of 25) were readily detectable 2 weeks after cell injection. Others became detectable later on. A study of intraocular tumor detection by fundoscopy, taking into consideration only the animals that were kept alive until the end of the experiment (constant n=17), showed that after week 5 the number of animals with detectable tumors remained stable (Figure 8).

Figure 8. Intraocular tumor detection by fundoscopy

Percentage of animals with detectable tumors by fundoscopy vs time. Only those animals that were kept alive until the end of the experiment are included in this study (n=17).

To evaluate tumor growth, animals were weekly classified in different stages according to the fundoscopic observations (Figure 9). As shown in Figure 9a, tumors were detectable 2 weeks after cell inoculation. The percentage of detectable tumors increases with time to become stable after 4-5 weeks.

On Figure 9b, only those animals with detectable tumors are represented to better appreciate the progression of the tumors through the different stages. In either 2 or 3 weeks after cell inoculation, tumors presented as localized nodules. By week 4 after cell injection, almost 50% of the tumors were involving 1 out of 4 quadrants of the retina. Some tumors were already involving 2 out of 4 quadrants of the retina in week 5. After week 5, most tumors involved more than 2 out of 4 quadrants and extraocular extension became more and more common.

Figure 9. Intraocular tumor growth evaluation by fundoscopy

The animals were classified in different stages according to the fundoscopic findings.

- a) Dark blue (stage 0) represents the percentage of animals with no detectable tumors. Only those animals in which fundoscopic evaluation was possible (with no opaque ocular media) were included in this study. b) Only those animals with detectable tumors were included in this study (stages 1-5).

Figure 7. Fundus photographs of rabbits' eyes

a) Normal eye fundus photo; b) Intraocular melanoma in a rabbit, 9 weeks after cell injection; c) Large tumor mass observed with the naked eye, 9 weeks after cell injection; d) Ciliary body melanoma observed through the pupil of a rabbit, 9 weeks after cell injection.

Ultrasound examination was performed to determine the echographic characteristics, location and final dimensions of the intraocular tumors *in vivo*. Using A-scan, intraocular tumors presented as solid lesions with low to medium, regular, internal reflectivity; all characteristic features of uveal melanoma (Figure 10). Using B-scan, intraocular tumors consisted mainly of large choroidal masses with a tendency for extraocular extension (Figure 10). Ultrasound revealed the presence of intraocular tumors in all 15 animals examined. Ciliary body involvement was detected in 4 of the 15 animals studied (27% of the cases) (Figure 10). Ten weeks after cell inoculation, tumors presented with an average length of 10.1 ± 1.5 mm (n=9), an average width of 10.0 ± 1.8 mm (n=9) and an average height of 7.5 ± 2.3 mm (n=15). In 6 cases, the tumor was so large that it exceeded the boundaries of the ultrasound field. In those eyes, the thickness of the tumor was the only measurement taken.

Figure 10. Ultrasound examination

a) and b) B-scan echograms showing mushroom-shaped lesions in the rabbits' eyes, 9 weeks after cell injection; c) B-scan echogram showing involvement of the ciliary body in a rabbit eye, 9 weeks after cell injection; d) A-scan echogram showing low-medium internal reflectivity.

5.2.2 Macroscopic and microscopic findings

The enucleated eyes were examined to determine the presence and morphology of the developed intraocular tumors post-mortem. Macroscopically, intraocular tumors were found in 82% of the animals (22 out of 27) (Figure 11). Ocular tumor development was observed in the first sacrificed animal, 1 week after cell injection. Histopathological evaluation of the enucleated eyes further revealed that tumors were present in 89% of the animals (24 out of 27). In this sense, post-mortem pathological examination of the enucleated eyes served to confirm the presence of intraocular tumors in those cases previously observed *in vivo* and to reveal tumors that were not detectable by fundoscopy. Melanomas were positively identified by immunostaining with a mAb to the melanoma-specific HMB-45 marker (Figure 12).

Intraocular tumor development was monitored throughout the experiment (Figure 12). Tumors grew diffusely within the choroid during the first 2 weeks. The site of cell injection could sometimes be identified as a thickened choroidal area. By week 3 and 4 after cell injection, tumors began to grow in height, adopting a nodular or dome-shaped configuration. From week 5 onwards, most of the eyes examined showed large tumors that varied in shape. The majority of these large tumors exhibited a mushroom-like appearance, with a considerable part of the tumor filling the vitreous cavity. At the end of the experiment, tumors completely filled the globe in 30% of the remaining animals. In those large tumors, viable tumor cells were located surrounding intratumoral blood vessels. Areas of necrosis distal to the intratumoral blood vessels were frequently observed, indicating the oxygen diffusion limitation within the tumor mass (Figure 13).

Figure 11. Progression of the intraocular tumors: macroscopic findings

a) Normal rabbit eye; b) Intraocular melanoma in a rabbit, 1 week after cell injection (magnification of the area indicated in Figure a); c) Intraocular melanoma in a rabbit, 6 weeks after cell injection; d) Intraocular melanoma filling the ocular cavity of the rabbit, 9 weeks after cell injection.

Figure 12. Progression of the intraocular tumors: microscopic findings

Histopathological sections showing tumors a) 10 days (H&E, 5x), b) 3 weeks (H&E, 5x) and c) 8 weeks after cell injection (H&E, 2.5x). Photomicrographs d), e) and f) are HMB-45 immunostained specimens a), b) and c), respectively.

Figure 13. Oxygen diffusion limitation within large tumor masses

a) Viable tumor cells surrounding an intratumoral blood vessel; b) The zones of necrosis are located distal to the intratumoral blood vessel.

5.2.3 Histopathological prognostic factors evaluation

The evaluation of uveal melanoma histopathological prognostic factors reflected the high aggressiveness of the ocular tumors (Figure 14). Tumors were of a mixed cell type, with predominantly epithelioid cells. Tumors were measured histopathologically in two dimensions. Table 4 shows the measurements of the tumors in height and diameter. Tumor height continuously increased during the first weeks. Six weeks after cell inoculation tumor height stabilized, reaching an average value of 4.6 ± 1.1 mm. The growth of the tumors in height is plotted in Figure 15.

<i>WEEK #</i>	<i>HEIGHT (mm)</i>	<i>LENGTH (mm)</i>
0	0	0
1	0.5	7.5
2	0.5	13.5
3	2.0	13.0
4	3.0	12.0
5	4.0	10.5
Thereafter	4.6 ± 1.1	11.6 ± 3.6

Table 4. Tumor dimensions by histopathology

Figure 15. Tumor growth in height

Six weeks after cell inoculation tumor height stabilized. The average height and standard deviation of the tumors after week 5 are shown.

A marked lymphocytic infiltration was found within the melanoma masses. With the exception of tumors from week 1, which showed less than 100 TIL per 20 HPF, all tumors had more than 100 TIL per 20 HPF and they were classified as belonging to the “high lymphocytic” group. A few vascular loops were observed in week 2 and 3 tumors. From week 4 onwards, all tumors exhibited a high number of vascular loops. The mean number of mitotic figures seen in 10 HPF was 48.7 ± 13.9 ($n=16$). A significantly lower value was obtained for week 1 tumors (30.5). The MTLNI measured in 25 HPF was 0.44 ± 0.04 ($n=16$). Again, a significantly lower value was obtained for week 1 tumors (0.32). Extraocular extension was consistently observed in tumors after week 4. At the end of the experiment, the majority of the remaining animals presented with extrascleral extension (90%).

Figure 14. Evaluation of the histopathological prognostic factors

a) Tumor of mixed cell type with predominantly epithelioid cells (H&E, 40x); b) Vascular loops (PAS, 40x); c) Mitotic activity (PAS, 20x). Arrows indicate normal bipolar and atypical tripolar mitotic figures; d) Cytomorphometry. Arrows indicate large nucleoli (H&E, 40x); e) Tumor infiltrating lymphocytes (H&E, 40x); f) Extraocular extension (H&E, 2.5x).

5.2.4 Culture of intraocular tumor cells

We were able to isolate and reculture cells from 16 intraocular tumors. Cell cultures were positively immunostained using Melan A and Tyrosinase mAb (Figure 16).

5.3 Malignant cell dissemination

5.3.1 Culture of circulating malignant cells

We successfully isolated CMC from blood samples obtained at the moment of euthanasia. Colonies, usually 2 or 3 per sample, were observed 2 weeks following the seeding of the mononuclear layers. The cells were positively identified as uveal melanoma cells through immunostaining using HMB-45, Melan A and Tyrosinase mAbs (Figure 17). *In vitro* proliferating CMC were first isolated 6 weeks after cell inoculation when we first attempted to culture mononuclear cells from the blood. CMC were detected in 5 of 19 (26%) cultured blood samples. Examination of cultured CMC grown on coverslips revealed that the cellular morphology resembled that of the original 92.1 human uveal melanoma cell line. CMC were kept frozen for future studies.

Figure 16. Cultured intraocular tumor cells isolated from rabbit eyes

a) Cytospin immunostained with Melan A monoclonal antibody (40x); b) Cytospin immunostained with Tyrosinase monoclonal antibody (40x).

Figure 17. Cultured circulating malignant cells isolated from rabbits' blood

a) and b) CMC grown in coverslip were immunostained using the HMB-45 monoclonal antibody (10x and 40x, respectively); c) and d) Cytospins immunostained with Melan A monoclonal antibody (10x and 40x, respectively); e) and f) Cytospins immunostained with Tyrosinase monoclonal antibody (10x and 40x, respectively).

5.3.2 RT-PCR as a potential tool for CMC detection

In order to evaluate RT-PCR as a potential tool for early detection of tumor cell dissemination, we investigated the ability of this technique to detect human uveal melanoma cells spiked into rabbits' blood samples. The sensitivity of RT-PCR and nested RT-PCR was also determined in this study.

Total RNA extracted from mononuclear cells isolated from blood samples were shown to have a high level of purity as determined spectrophotometrically. A₂₆₀/A₂₈₀ ratios typically ranged between 1.9 and 2.0. The final concentrations of RNA extracted ranged between 0.1 and 0.3 mg/ml.

RT-PCR, as well as nested RT-PCR, allowed for the detection of human uveal melanoma cells spiked into rabbit peripheral blood samples. Reverse transcription and PCR amplification of either Tyrosinase or Melan A melanoma messenger RNA was possible using this technique. Re-amplification (nested PCR) of the PCR products was also achieved giving rise to the expected size re-amplification products (Figure 18). Negative control samples showed no amplification products.

The limit of detection was 10^5 spiked cells/ml of blood by RT-PCR. A remarkable improvement in sensitivity was achieved using nested RT-PCR. We were able to detect the presence of 10^2 spiked cells/ml of blood using the latter technique. No difference in sensitivity between the melanoma markers evaluated was observed.

Figure 18. Detection of uveal melanoma cells by RT-PCR

The product of RT-PCR and nested RT-PCR are visualized in ethidium bromide stained 1% agarose gels. Amplification products for two specific melanoma markers (Tyrosinase and Melan A) are shown. The level of detection is indicated on the gels. Lanes. M: Molecular weight marker; C₁: Positive control for first amplification round; C₂: Positive control for second amplification round; 1: Negative control; 2: 10 cells/mL; 3: 10² cells/mL; 4: 10³ cells/mL; 5: 10⁴ cells/mL; 6: 10⁵ cells/mL; 7: 10⁶ cells/mL

5.4 Metastasis

5.4.1 Macroscopic and microscopic findings

Gross examination of the lungs revealed multiple nodular lesions compatible with metastasis 7 weeks after surgery (Figure 19). Lung metastases were macroscopically observed in 41% of the animals remaining at the end of the experiment. They presented as firm elevated lesions of less than one centimeter in diameter. No macroscopic metastatic lesions were observed in either the liver or the kidneys of the rabbits.

Microscopic pulmonary metastatic foci were first observed 4 weeks after cell injection. By the end of the experiment, all remaining animals (100%) showed histopathologically confirmed lung metastasis (Figure 20). Metastatic lesions were HMB-45 positive (Figure 21). The lesions usually presented as multiple subpleural micronodules surrounded by a peripheral rim of lymphocytes. In some cases, areas of diffuse infiltrate were also observed probably causing the breathing difficulties observed in some of the animals (Figure 20). Interestingly, micrometastatic lesions were found in the liver of 3 out of 17 (17.6%) animals remaining at the end of the 10th week (Figure 22). Some inflammatory cells could also be found within the lesions. The microarchitecture of the liver was well preserved. No other pathological findings were observed.

5.4.2 Culture of metastatic cells

Cells from 4 out of 6 metastatic lung lesions were successfully isolated and grown *in vitro*. These rapidly proliferating metastatic cells were positively immunostained with specific antibodies against melanoma markers (Figure 23). Metastatic cells were preserved frozen for future experiments.

Figure 19. Metastases: macroscopic findings

a) Normal rabbit lungs; b) Gross examination of a rabbit lung, 7 weeks after cell injection. Arrows indicate multiple nodules compatible with metastasis. No metastatic lesions were observed in c) the livers or d) the kidneys of the animals.

Figure 20. Lung metastases: microscopic findings

a) and b) Photomicrographs of a H&E stained specimen showing a subpleural micronodule (10x and 40x respectively); c) and d) Metastatic lesion in a rabbit lung (10x and 40x respectively); e) Metastatic nodule with peripheral rim of lymphocytes (H&E, 20x); f) Photomicrograph of a H&E stained specimen showing a diffuse infiltrate in a rabbit lung (5x).

Figure 21. Photomicrographs of HMB-45 immunostained lung metastatic nodules
a) Subpleural metastasis (10x); b) and c) Lung micrometastatic nodule (10x and 40x, respectively).

Figure 22. Liver metastases: microscopic findings

a), b) and c) Metastatic foci in the rabbit liver (H&E; 10x, 20x and 40x, respectively); d), e) and f) Malignant cells invading the rabbit liver (H&E; 10x, 20x and 40x, respectively).

Figure 23. Cultured metastatic cells isolated from rabbits' lung lesions

a) and b) Cultured metastatic uveal melanoma cells grown in coverslip were immunostained using the HMB-45 monoclonal antibody (20x and 40x, respectively); c) Cytospin immunostained with Melan A monoclonal antibody (20x); d) Cytospin immunostained with Tyrosinase monoclonal antibody (20x).

Chapter 6

DISCUSSION

In this thesis work, a detailed characterization of uveal melanoma progression including tumor development, malignant cell dissemination and metastasis was performed using the albino rabbit model.

By using the strategy of sacrificing one animal per week, we were able to histopathologically demonstrate that the disease progresses in a sequential manner. This strategy represents an advantage compared to previous animal models. During the first week, the injected human uveal melanoma cells adapted to grow in the new environment. Subsequently, intraocular tumors developed and grew rapidly until week 5. After week 5, tumors extended extraocularly and presented with the worst prognostic factors. Systemic dissemination occurred leading to the development of metastasis. By the time metastasis was evident, tumors were already occupying most of the ocular cavity. In fact, the time lung metastasis was observed coincided with the extension of the tumors extraocularly.

One of the main limitations of this animal model is the need for continuous immunosuppression to allow for efficient tumor development (Ordonez, Saornil et al. 2002). Particularly, the potentially severe adverse reactions that can be observed due to CsA toxicity, may limit the length of the experiment and thus the time in which metastatic disease can fully develop. Moreover, short-time experiments may limit the time in which therapeutic effects can be demonstrated when using the model in pre-clinical trials. In addition, CsA side effects may mask therapeutic benefits. Careful dosage regimen selection minimizes CsA side effects (Blanco, Saornil et al. 2000) and consequently results in improved models. We obtained a high survival rate, with 90% of the animals surviving until the end of the experiment. We believe that the initial animal weight (more

than 3 kg) and the efficient animal care played a key role in this outcome. The selection criteria for the weekly euthanization may also have had an influence in the overall survival rate. One of the major side effects of CsA is weight loss. In this animal model, the rabbits maintained their weight throughout the experiment, indicating that CsA doses were well adjusted.

Fundoscopy allowed for the evaluation of tumor presence and growth *in vivo*. Nevertheless, the use of funduscopy for the evaluation of tumor take rate may have certain limitations. The detection of intraocular tumors was not possible in 22% of the animals by this method, either because the animals presented with severe intraocular hemorrhage or retinal detachment or because the tumors were not clinically visible. In those animals, later post-mortem pathological examination of the enucleated eyes demonstrated the presence of a tumor. Alternatively, ultrasound could be used for the evaluation of tumor development *in vivo* (Blanco, Oliver et al. 2003). Ultrasound is particularly useful in cases with opaque ocular media and extraocular tumors. In addition, it allows for a more accurate determination of tumor location and dimensions.

In this model, ultrasound provided an accurate measurement of the tumor size. However, by the time ultrasound examination was performed, the tumors were so large that their specific location was not possible to determine. The location of the tumor is considered a prognostic factor. Ciliary body melanomas are associated with poor prognosis. They tend to be large at the time of diagnosis, carrying a high mortality rate. In this animal model, ultrasound detected a clear involvement of the ciliary body in 4 of the 15 animals examined. In the other cases, involvement of the ciliary body most likely

occurred, but it was hard to confirm. Nevertheless, the value of ciliary body involvement as a prognostic factor at this stage is not significant since metastatic disease was observed in all 15 animals.

Intraocular tumors successfully developed in the majority of the animals. The cell injection procedure into the rabbits' eyes yielded a take-rate of 89% as revealed by macroscopic and microscopic studies performed in the enucleated eyes. In 3 cases, the tumors could not be found microscopically, although they have been previously observed macroscopically during autopsy. It is very well possible that these tumors were lost while cutting eyes in fresh. Eyes were cut in fresh (not fixed) in order to culture cells from the intraocular tumors. The intraocular tumors in 2 of these 3 cases were also observed *in vivo* by fundoscopy and ultrasound.

Interestingly, the 11% of animals (3 cases) that did not developed intraocular tumors (not observed neither by fundoscopy, ultrasound nor pathologically) presented metastatic disease. All 3 cases belonged to the last week of the experiment. These metastases most likely occurred by direct injection of the tumor cells into a choroidal vessel instead of into the suprachoroidal space, a phenomenon known as "seeding at implantation" (Gadeholt-Gothlin and Gothlin 1995). The suprachoroidal space is a virtual space, and the choroid, the layer that lies just below, is extremely rich in blood vessels. Therefore, the accidental infusion of the human uveal melanoma cells into the vessels could have caused these metastases artificially. Although these animals are not useful for the study of disease progression from tumorigenesis to metastasis, they could be used for the study of metastatic disease.

The biological characteristics of the tumors developed in the rabbit eye closely resembled those of the disease in human beings. The tumors displayed similarities in cytoarchitecture, morphology, phenotype and microcirculation patterns to human uveal melanoma. Standardized A-scan ultrasonography showed the expected echographic characteristics for uveal melanoma. The tumors of the model were, however, much more aggressive. Consequently the disease progressed and spread over a shorter period of time, a desirable feature in an animal model of metastatic disease. The evaluation of histopathological prognostic factors of uveal melanoma reflected the highly aggressive nature of these tumors. Tumors were mainly composed of the most invasive cell type. They grew rapidly and extended beyond the sclera. Most values obtained, including TIL, mitotic activity and MTLNI, were considerably higher than those commonly observed in patients with uveal melanoma. Lesions were well vascularized since the first weeks of the experiment, although the microvascularization accentuated as the tumor size increased. For every prognostic factor evaluated a significantly different value was obtained for week 1 tumors, indicating that the cells were still adapting to grow *in vivo*.

To the best of our knowledge, this is the first time that the isolation and/or detection of CMC is reported in a uveal melanoma animal model. Our results may have important consequences for the study of disease dissemination since the presence of CMC in peripheral blood represents an early step of malignant cell dissemination. We demonstrated that viable uveal melanoma cells indeed circulate in the peripheral blood of the animals and they can be isolated and grown *in vitro*. Re-cultured CMC maintained the expression of specific melanoma markers. Further characterization studies are underway to attempt the identification of changes that intraocular tumor cells might have undergone

allowing them to escape from the eye. Additionally, the detection of these cells encourages the development of new diagnostic strategies aimed to recognize early stages of malignant cell dissemination and monitor patients that are at high risk of developing metastasis.

We first attempted to culture CMC in week 6. By that time, intraocular tumors were large and already extended beyond the sclera, and metastatic disease was already present. Therefore, whether the detection of CMC would precede the development of metastasis, remains unclear. Moreover, a correlation between tumor size or other prognostic factors and the presence of CMC could not be established. In future experiments, blood samples should be processed beginning in week 1 to determine the exact time when these viable cells can be detected in circulation and to correlate their presence with uveal melanoma prognostic factors and metastasis development.

In addition, we explored the potential of PCR to detect CMC in rabbit blood samples. We demonstrated that RT-PCR could be a useful tool, provided the disseminated cells maintain the phenotype of the original inoculated cell line *in vivo*, as they did in *in vitro* studies. Both markers (Tyrosinase and Melan A) proved to be equally useful to detect spiked melanoma cells into rabbits' blood. The nested RT-PCR method proved to be 1000-fold more sensitive than the non-nested RT-PCR method.

Although both CMC detection methods proved to be useful, only by culturing mononuclear blood cells, viable CMC can be identified. Since only viable cells have the potential to metastasize, the strategy of culturing will indicate a more realistic risk for

metastasis development. Ideally, in order to combine the sensitivity and specificity of RT-PCR with the ability to identify viable cells of cell culture techniques, both methods should be employed.

A good experimental model for the study of metastatic uveal melanoma should present metastasis in a high proportion of animals (Blanco, Caissie et al. 2004). The high rate of lung metastasis achieved encourages the evaluation of novel systemic therapeutic approaches using this model. In addition, hepatic metastasis was found in 18% of the remaining rabbits in the last week of the experiments. To the best of our knowledge, liver metastasis using this particular experimental model or similar ones has not been reported to date. It should be pointed out that the liver is the preferential, and often the only site of metastasis in patients with uveal melanoma. Our findings indicate that this animal model provides a unique opportunity to study not only the metastatic cascade by itself but also the organ microenvironment where metastasis normally occurs in human beings. Furthermore, this model will allow for the study of therapies targeted against metastasis in a more representative context. Extending the length of the experiment may result in a higher rate of liver metastasis. We believe that the prolongation of the experiment is feasible since low mortality rates were observed and the animals reached the end of the experiment in good condition.

Chapter 7

SUMMARY AND CONCLUSION

The management of uveal melanoma remains a clinical dilemma, which reflects our poor understanding of the disease. Although experimental models may never exactly replicate the disease in human beings, they provide useful tools that permit the study of specific aspects of the disease.

This study has advanced our understanding of the biology of uveal melanoma. We were able to document the progression of human uveal melanoma in this animal model, as well as to characterize the intraocular tumors and evaluate the prognostic factors related to them. Furthermore, the development of human uveal melanoma in the animals could be assessed using the same tools that are commonly used in patients with uveal melanoma. In addition, this experimental system demonstrated the presence of CMC in the peripheral blood of the animals. The detection of uveal melanoma cells in rabbits' blood was accomplished using two different strategies: cell culture and RT-PCR. Finally, the rate, time-course and site of metastatic disease were determined.

We are reporting a complete and unprecedented source of information intended to serve as a baseline for future experiments using this model. We believe that the albino rabbit model of uveal melanoma is a promising tool for the evaluation of new systemic therapeutic approaches and offers great potential for the development of methods for the early detection of disease spread. Progress made in these areas will ultimately be translated into clinical benefits for patients affected by uveal melanoma.

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APPENDIX

Uveal melanoma is the most common intra-ocular tumor in adults. The survival rate of this malignancy has not significantly increased over the last several decades despite the modalities of local treatment such as enucleation or irradiation. The disease is lethal (50 % of the patients die after 10 years from the time of diagnosis) due to a metastatic dissemination of malignant cells. This metastasis can be diagnosed only in late stages when no treatment is available. For that reason, the establishment of a sensitive method to detect circulating malignant cells in peripheral blood (cells that are able to produce metastases) would be crucial in order to detect metastases in early stages (subclinical metastases that may be treated) and improve the survival of the patients. The aim of this study is to detect specific melanoma proteins in the peripheral blood of rabbits that have had human uveal melanoma cells injected into the eye. Molecular biology techniques can be used to detect these melanoma proteins which may serve as sensitive and specific markers of uveal melanoma dissemination, and help establish a diagnosis in the early stages of the metastatic disease. In our previous animal model, metastases could be detected at the end of the 8-week experiment. However, we were not able to determine the time when metastasis first developed. In order to document the progression of the disease and to correlate the week of metastasis development with the detection of circulating malignant cells, one rabbit will be euthanized per week starting after the first week of the 10-week experiment. It is hoped that in the future the method of detecting melanoma cells in blood may be used with uveal melanoma patients to help detect early metastasis formation that could be successfully treated. This study will also give important information concerning the time course of metastasis development in this animal model and its relation to the presence of circulating malignant cells.

5 b) SPECIFIC OBJECTIVES OF THE STUDY: Summarize in point form the primary objectives of this study.

- A). In vivo: To detect, in different steps of tumoral development, specific melanoma proteins in the peripheral blood of rabbits in the experimental uveal melanoma model.
- B) In vivo: To determine the time course of metastasis development in this animal model.
- C). In vitro: To establish, by molecular biology techniques, the minimum number of tumor cells that is possible to be detected using specific proteins of melanoma cells. One human uveal melanoma cell line (92-1) will be used.
- D). To correlate the molecular biology results of the different steps of intraocular tumor development with metastasis development, as determined by histopathological and immunohistochemical findings of the uveal melanoma rabbit model.

5 c) Indicate if and how the current goals differ from those in last year's application.

The previous animal model (#4226) was an 8 week experiment, however, the proposed animal model will be a 10-week experiment in order to further study the development of metastasis. Although the euthanization of one rabbit per week has been a significant enough change to the previous uveal melanoma protocol (#4226) to have warranted the submission of a new application, it should be noted that the surgical procedure, and subsequent tissue and cellular analysis, will be virtually identical to the previous model of uveal melanoma (protocol #4226). In addition, the quality of animal follow-up will be enhanced as an animal health technician will be assisting us with this protocol.

5 d) List the section / subsection numbers where significant changes have been made

5 e) KEYWORDS: Using keywords only, list the procedures used on animals (e.g. anaesthesia, breeding colony, injection IP, gavage, drug administration, major survival surgery, euthanasia by exsanguination, behavioural studies). For a more complete list of suggested keywords refer to Appendix 1 of the Guidelines (www.mcgill.ca/rgo/animal).

>1) immunosuppression 2) survival surgery: general and local anaesthesia 3) implantation of tumor cells 4) analgesia 5) daily observation of animal health 6) weekly observation of the eye (presence of tumors) 7) weekly sedation/physical restraint/blood collection 8) exsanguination with anaesthesia 9) tissue collection

6. Animals Use data for CCAC

6 a) Purpose of Animal Use (Check one):

1. ☐ Studies of a fundamental nature/basic research
 2. ☒ Studies for medical purposes relating to human/animal diseases/disorders
 3. ☐ Regulatory testing
 4. ☐ Development of products/appliances for human/veterinary medicine
5. If for Teaching, use the Animal Use Protocol form for Teaching (www.mcgill.ca/rgo/animal)

6 b) Will field studies be conducted? NO ☒ YES ☐ If yes, complete "Field Study Form"

Will the project involve the generation of genetically altered animals? NO ☒ YES ☐ If yes, complete SOP #5

Will the project involve breeding animals? NO ☒ YES ☐ If breeding transgenics/knockouts, complete SOP #4

7 Animal Data

a) Please justify the need for live animals versus alternate methods (e.g. tissue culture, computer simulation)

This study needs to observe the metastatic spread of tumor cells through blood and eventual implantation at a secondary site. Tumor metastasis is a major complication of cancer development that can only occur in live animals.

b) Describe the characteristics of the animal species selected that justifies its use in the proposed study (consider characteristics such as body size, species, strain, data from previous studies or unique anatomic/physiological features)

Albino rabbits have been chosen because ocular examination of melanotic tumors in these animals is more accurate than that in pigmented rabbits. Also, the almost identical size of ocular structure in this species compared to humans, allows for tumor cell implantation during surgery and eye examination upon follow-ups. The eyes of other species such as rodents are too small for the surgical procedure of tumor cell implantation, as well as eye examinations upon follow-ups to monitor uveal melanoma development. The sex election comes from the hormonal changes females undergo. This effect may interact with the experiment. Finally, animals weighing 3 kg. are more resistant to the toxic action of the immunosuppressant cyclosporin A.

c) Description of animals

Quality Control Assurance: To prevent introduction of infectious diseases into animal facilities, a health status report or veterinary inspection certificate may be required prior to receiving animals from all non-commercial sources or from commercial sources whose animal health status is unknown or questionable. Quarantine and further testing may be required for these animals.

If more than 6 columns are needed, please attach another page

	Sp/strain 1	Sp/strain 2	Sp/strain 3	Sp/strain 4	Sp/strain 5	Sp/strain 6
Species	New Zealand Rabbits					
Supplier/Source	Charles River					
Strain	Albino					
Sex	Male					
Age/Wt	3months/3kg					
# To be purchased	28					
# Produced by in-house breeding	N/A					
# Other (e.g. field studies)	N/A					
#needed at one time	All					
# per cage	1					
TOTAL# /YEAR	28					

7 d) Justification of Animal Usage: BASED ON THE EXPERIMENTAL OBJECTIVES OF THE PROJECT, describe the number of animals required for one year. Include information on experimental and control groups, # per group, and failure rates. For breeding, specify how many adults are used, number of offspring produced, and how many offspring are used in experimental procedures. Use the table below when applicable. The arithmetic explaining how the total of animals for each column in the table above is calculated should be made clear. Space will expand as needed.

Twenty five animals are required for this study. All animals will receive the same cyclosporin A dosing regimen. Three additional animals will be needed in case of surgery failure (vitrectomy upon tumor cell implantation) according to previous studies. The animal model to be used is referenced in: 1) Saornil et al. Growth of a human uveal melanoma cell line in rabbits immunosuppressed with different doses of cyclosporin A. IOVS 1996; 37:S244. 2) Domingo et al. Effect of cyclosporin A on the development of experimental choroidal tumors from uveal melanoma cells in pigmented rabbits. IOVS 1997; 38: S802.3) Morilla-Grasa A et al. An animal model of primary and metastatic human uveal melanoma: Melan A and HMB-45 expressivity. IOVS 1999; 40:S246. 4) Blanco G et al. Efficacy of five human melanocytic cell lines in experimental rabbit choroidal melanoma. IOVS 1999; 40: S247.

In the preliminary study of Saornil et al., two groups of 20 rabbits each were used to assess the efficacy of two fixed doses of cyclosporin A to allow for tumor formation and metastasis. (1) The second study by Domingo et al. showed that varying doses of cyclosporin A are required throughout the experiment. This group used 3 groups of 15 rabbits per group.

The uveal melanoma cell line, 92-1, has been selected based on its high in vitro proliferation rate and its metastatic potential. This cell line was used in our previous 8-week animal model of uveal melanoma (protocol #4226) in which its capacity to cause tumor formation and its metastatic potential was documented. The 92-1 cell line was also used in the other previous animal models of uveal melanoma including that of Blanco G et al. Efficacy of five human melanocytic cell lines in experimental rabbit choroidal melanoma. IOVS 1999; 40: S247. The proposed animal model will be a 10-week experiment to further study the development of metastasis.

Considering the mortality rates due to Cyclosporin A (25-30%) and the fact that one animal per week will be euthanized, we will require 25 animals to ensure that at least 10 live animals will remain at the end of the 10-week experiment. The 92-1 cell line has been shown to have 100% tumorigenic potential and 50% metastatic potential. Therefore, 10 rabbits are required in order to accurately compare the metastatic potential of 92-1 in this model (with expected metastases in 4-5 of 10 rabbits) and its metastatic potential determined from previous models.

The following table may help you explain the animal numbers listed in the 7c table:

(table will expand as needed)	Sp/strain 1	Sp/strain 2	Sp/strain 3	Sp/strain 4	Sp/strain 5	Sp/strain 6
Test Agents or procedures	1					
# of animals per group	25					
Dosage and/or route of administration	IM					
# of endpoints	1					
Other variables (sex, genotypes...)						
Total number of animals per year	25 (+3 in case of surgical failure) 28 total					

8. Animal Husbandry and Care

8 a) If project involves non-standard cages, diet and/or handling, please specify

N/A

8 b) Is there any component to the proposed procedures which will result in immunosuppression or decreased immune function (e.g. stress, radiation, steroids, chemotherapeutics, genetic modification of the immune system)?

NO ☐ YES ☒ if yes, specify: Cyclosporin A

8 c) Indicate area(s) where animal use procedures will be conducted:

Building: Lyman Duff Room: 7th Floor

Indicate area(s) all facilities where animals will be housed:

Building: Lyman Duff Room: 7th Floor

If animal housing and animal use are in different locations, briefly describe procedures for transporting animals:
N/A

9. Standard Operating Procedures (SOPs)

Complete this section if you plan to use any of the UACC SOPs listed below. IT IS UACC POLICY THAT THESE SOPs BE USED WHEN APPLICABLE. Any proposed variation of the SOPs must be described and justified. The Standard Operating Procedures can be found at the UACC website at www.mcgill.ca/rgo/animal. The completed and signed SOP form must be attached to the protocol.

Check all SOPs that will be used:

Blood Collection UACC#1	☐	Collection of Amphibian Oocytes UACC#9	☐
Anaesthesia in rodents UACC#2	☐	Rodent Survival Surgery UACC#10	☐
Analgesia in rodents UACC#3	☒	Anaesthesia & Analgesia Neonatal Rodents UACC#11	☐
Breeding transgenics/knockouts UACC#4	☐	Stereotaxic Survival Surgery in Rodents UACC#12	☐
Transgenic Generation UACC#5	☐	Euthanasia of Adult & Neonatal Rodents UACC#13	☐
Knockout/in Generation UACC#6	☐	Field Studies Form	☐
Production of Monoclonal Antibodies UACC#7	☐	Phenotype Disclosure Form	☐
Production of Polyclonal Antibodies UACC#8	☐	Other, specify:	☐

10. Description of Procedures

10 a) IF A PROCEDURE IS COVERED BY AN SOP, WRITE "AS PER SOP", NO FURTHER DETAIL IS REQUIRED.

FOR EACH EXPERIMENTAL GROUP, DESCRIBE ALL PROCEDURES AND TECHNIQUES, WHICH ARE NOT PART OF THE SOPs, IN THE ORDER IN WHICH THEY WILL BE PERFORMED – surgical procedures, immunizations, behavioural tests, immobilization and restraint, food/water deprivation, requirements for post-operative care, sample collection, substance administration, special monitoring, etc. Appendix 2 of the Guidelines (www.mcgill.ca/rgo/animal) provides a sample list of points that should be addressed in this section.

92-1 uveal melanoma cells have been tested for Mycoplasma and they are infection free. In addition, they are observed twice weekly for any bacterial or fungal infections. The same cell line is used routinely in the lab for in vitro studies and it was used in our previously approved animal model of uveal melanoma (protocol #4226). No potential health risks to humans or animals are anticipated-the same cell line has been used in all previous animal models of uveal melanoma described above.

Surgical procedure: aseptic conditions

1. Right eye of the rabbit will be dilated 10 minutes before operation using drops of phenylephrine hydrochloride and tropicamide (only for ophthalmoscopic examination).
2. The animal will be anaesthetized using ketamine-xylazine.
3. A fundoscopic examination of the eye will be made using an indirect binocular ophthalmoscope in order to rule out any ocular pathology.
4. Ocular anaesthetic (3-4 drops) proparacaine hydrochloride will be applied topically in the right eye of the animal one minute before the operation.
5. Superior rectus muscle and inferior rectus muscle with a 4/0 suture will be taken using a needle holder to move the eye easily.
6. Temporal superior conjunctiva will be separated from the sclera in order to clean the area of the sclera where the incision will be made.
7. One sclerotomy of 2-3 mm using an ophthalmic knife will be made. The incision must be until the suprachoroidal space (brown colour). If a vitrectomy (vitreous humor leaving from the incision) is made the animal cannot be used for the experiment and one of the spare animals will be used.
8. A paracentesis (to take out aqueous humor from anterior chamber) will be made using an insulin-like syringe. The extracted volume (0.2-0.5 mL) will rinse the eye surface.
9. A tunelizer will be used to work a tunnel in the suprachoroidal space by the incision already made.
10. The incision ends will be sewed with the same reabsorbible 7/0 suture (forming a U) using a needle holder.
11. The insulin-like syringe with the canulla containing 1million cells in 0.2 mL of culture medium will be introduced in the tunnel. a) Introduce the canulla b) Tighten the sutures c) Inject the cells d) Knot the suture just when the canulla will be extracted.
12. Antibiotic (erythromycin) ointment will be applied on the eye. If one animal misses injection and a vitrectomy is produced, the animal will be euthanized, however, in our experience the probability of this occurring is less than 5 %.
13. In the two first days post-op 0.02 mg/Kg of buprenorphine will be provided twice a day (every twelve hours) or as determined

necessary by the animal health technician and veterinarian (see SOP).

Animal follow-up:-

1. All 25 animals will receive the same dose of cyclosporin A. In all rabbits cyclosporine A will be administered intramuscularly. Animals will receive 15mg/kg of cyclosporin for 3 days prior to surgery and the first 4 weeks following surgery, and then 10 mg/kg for the remaining 6 weeks.

2. The animal health technician will be available to help with post-op follow-up every day for the first two weeks and then twice a week thereafter or as determined necessary by the animal health technician and the veterinarian. Every day, animals will be weighed in order to correct the intramuscular cyclosporine A doses. The doses of cyclosporin A required to allow for tumor growth were selected based on findings obtained from previous animal models of uveal melanoma and an extensive literature review looking at intramuscular cyclosporine A administration in animal models, as well as consultation with a veterinarian. The highest dose of cyclosporin A to be administered is 15mg/kg for 3 days and 4 weeks. All animals will be monitored for secondary effects of the immunosuppressant and the doses will be adjusted according to the veterinarian's recommendations as required.

3. Daily evaluation of the rabbits will be conducted to identify any secondary effects of cyclosporine A (weight loss, diarrhea, vomiting). The animals will also be monitored for signs of ocular infection/inflammation that would be treated with trimethoprim-sulphadiazine. The veterinarian will be advised again of any infection not responsive to standard treatment. Euthanasia will be practised if the veterinarian considers it to be necessary.

4. Fundoscopic evaluations will be performed 24 hours after the initial operation and every week after that for the remainder of the experiment (10 weeks).

5. Results will be categorized according to the following criteria: * 0: No tumor * 1: nodule size as papillar diameter or less * 2: tumor size as 1 eye quadrant. * 3: tumor size as 2 eye quadrants * 4: tumor size as 3-4 eye quadrants. * 5: tumor extrascleral extension and/or metastatic disease.

6. Photographic documentation of the ophthalmologic findings.

7. As this project is going to be done in areas with a high rate of asepsia, (Lyman Duff Animal Care facilities) and as it is going to use clean surgical procedures, no prophylaxis was recommended by Dr Matsumiya, veterinarian from the Animal Resources Center of the Royal Victoria Hospital. If infection symptoms are found, a subcutaneous daily dose of 30 mg/Kg of Trimethoprim-sulphadiazine will be administered.

8. 4 mL of peripheral blood (central ear artery) will be extracted from the rabbits every week. The sedative hypnorm will be administered intramuscularly prior to the blood extraction. In order to protect the animals from excessive movements during this procedure, animals will be placed in restraining cages. Blood samples collected prior to surgery will serve as negative controls. Malignant cells will be detected in the specific week they start to circulate.

9. In our previous animal model, metastases could be detected at the end of the 8-week experiment. In order to document the progression of the disease and to correlate the week of the metastasis development with the detection of circulating malignant cells, one rabbit will be euthanized per week (exanguination with anaesthesia) starting after the first week of the 10-week experiment. Autopsy and tissue collection (eyes, liver, lungs and any other tissue possibly infiltrated by tumor) will be performed on each euthanized rabbit. The remaining rabbits will be sacrificed at the end of the 10th week.

10 b) Experimental endpoint - for each experimental group indicate survival time

One rabbit will be euthanized per week (exanguination with anaesthesia) starting after the first week of the 10-week experiment. The remaining rabbits will be sacrificed at the end of the 10th week.

10 c) Clinical endpoint - describe the conditions, complications, and criteria (e.g. >20% weight loss, maximum tumour size, vocalizing, lack of grooming) that would lead to euthanasia of an animal before the expected completion of the experiment (specify per species and project if multiple projects involved)

A veterinarian (Dr. Matsumiya) would be contacted if there was >10% weight loss, lack of grooming or eating or drinking/vomiting/diarrhea/decrease in strength, due to immunosuppression and its secondary effects. In the event that some of the rabbits may have to be euthanized prematurely (as determined by the veterinarian), the animals will undergo exanguination with anesthesia.

10 d) Specify person(s) who will be responsible for animal monitoring and post-operative care (must also be listed in section 4)

Name: Paula Blanco
Jean Claude Marshall

Deanna Collin

Phone #: 398.7192 ext.: 00384
398-7192 ext: 00384

398-3510

10 e) Pre Anesthetic/Anaesthetic/Analgesic Agents. List all drugs that will be used to minimize pain, distress or discomfort. Table will expand as needed.

Species	Agent	Dosage (mg/kg)	Total volume(ml) per administration	Route	Frequency
Total volume per administration depending on weight of the rabbits (weighed daily). For example volumes have been calculated below for rabbits weighing 3kg.					
Albino Rabbits	1) Ketamine-xylazine	100mg/ml ketamine 35mg/kg + 20mg/ml xylazine 5mg/kg	{1.05ml} {0.75ml}	IM	10-15 minutes pre- surgery
	2) Hypnorm stock fentanyl 0.315 mg/ml and fluanisone 10 mg/ml	0.2-0.5ml/kg	{0.6-1.5ml}	IM	pre-blood collection
	3) Buprenorphine 0.3mg/ml	0.02-0.05 mg/kg	{0.2-0.5ml}	IM	post-surgery and every 12 hours after for 2 days (as per SOP)

10 f) Administration of ALL other substances: List all non-anesthetic agents under study in the experimental component of the protocol, including but not limited to drugs, infectious agents, viruses. Table will expand as needed.

Species	Agent	Dosage (mg/kg)	Total volume(ml) per administration	Route	Frequency
Volumens have been calculated below for rabbits weighing 3kg.					
Albino rabbit	1) Cyclosporin A	First 4 weeks + 3 days: 15 mg/kg	0.9ml	Intramuscular	Daily
		Last 6 weeks: 10mg/kg	0.6ml	Intramuscular	Daily
	2) Phenylephrine hydrochloride (Mydfrin) 2.5%			1-2 eye drops	each ophthalmic examination
	3) tropicamide (Mydriacyl) 1%			1- eye drops	each ophtalmic examination
	3) Erythromycin ointment 5mg/g			topical during surgery	
	4) ocular anaesthetic-proparacaine hydrochloride 0.5% (Alcaine)			2-3 drops pre-surgery	
	5) Trimethoprim-sulphadiazine	(stock) 40mg/ml 200 mg/ml	30mg/kg	SC	daily after infection signs

10. Method of Euthanasia	
Specify Species	
rabbits	☐ Anaesthetic overdose, list agent/dose/route:
	☒ Exsanguination with anaesthesia, list agent/dose/route: Ketamine-Xylacine stock 100mg/ml ketamine 35mg/kg (X3) (1.05ml) stock 20mg/ml xylacine 5mg/kg (X3) (0.75ml) intramuscularly
	☐ Decapitation without anaesthesia *
	☐ Decapitation with anaesthesia, list agent/dose/route:
	☐ Cervical dislocation without anaesthesia *
	☐ Cervical dislocation with anaesthesia, list agent/dose/route:
	☐ CO ₂ chamber
rabbits	☐ Other, specify:
	☐ Not applicable, explain:
* For physical method of euthanasia without anaesthesia, please justify:	

11. Category of Invasiveness:	B ☐	C ☐	D ☒	E ☐
Categories of Invasiveness (from the CCAC <i>Categories of Invasiveness in Animal Experiments</i>). Please refer to this document for a more detailed description of categories.				
Category A: Studies or experiments on most invertebrates or no entire living material.				
Category B: Studies or experiments causing little or no discomfort or stress. <i>These might include holding animals captive, injection, percutaneous blood sampling, accepted euthanasia for tissue harvest, acute non-survival experiments in which the animals are completely anaesthetized.</i>				
Category C: Studies or experiments involving minor stress or pain of short duration. <i>These might include cannulation or catheterizations of blood vessels or body cavities under anaesthesia, minor surgery under anaesthesia, such as biopsy; short periods of restraint, overnight food and/or water deprivation which exceed periods of abstinence in nature; behavioural experiments on conscious animals that involve short-term stressful restraint.</i>				
Category D: Studies or experiments that involve moderate to severe distress or discomfort. <i>These might include major surgery under anaesthesia with subsequent recovery, prolonged (several hours or more) periods of physical restraint; induction of behavioural stresses, immunization with complete Freund's adjuvant, application of noxious stimuli, procedures that produce pain, production of transgenics (in accordance with University policy).</i>				
Category E: Procedures that involve inflicting severe pain, near, at or above the pain threshold of unanaesthetized, conscious animals. <i>Not confined to but may include exposure to noxious stimuli or agents whose effects are unknown; exposure to drugs or chemicals at levels that (may) markedly impair physiological systems and which cause death, severe pain or extreme distress or physical trauma on unanaesthetized animals. According to University policy, E level studies are not permitted.</i>				

12. Potential Hazards to Personnel and Animals. It is the responsibility of the investigator to obtain the necessary Biohazard and/or Radiation safety permits before this protocol is submitted for review.			
A copy of these certificates must be attached, if applicable.			
No hazardous materials will be used in this study: ☐			
12 a) Indicate which of the following will be used in animals:			
☒ Toxic chemicals	☐ Radioisotopes	☐ Carcinogens	☐ Infectious agents ☒ Transplantable tumours
12 b) Complete the following table for each agent to be used (use additional page as required):			
Agent name	human uveal melanoma cells	Cyclosporin A	
Dosage	one million cells per animal	15mg/kg 4weeks+3days and 10mg/kg 6weeks	
Route of administration	ocular injection	intramuscular	
Frequency of administration	once at start of experiment	daily	

4. RESEARCH PERSONNEL: (attach additional sheets if preferred)

Name	Department	Check appropriate classification				
		Investigator	Technician & research assistant	Student		Fellow
				Under-graduate	Graduate	
Jean-Claude Marshall	Pathology	☐	☐	☐	☒	☐
Paula Blanco	Pathology	☐	☐	☐	☒	☐
Emilia Antecka		☐	☒	☐	☐	☐
		☐	☐	☐	☐	☐
		☐	☐	☐	☐	☐

5. EMERGENCY: Person(s) designated to handle emergencies

Name: Jean-Claude Marshall

Phone No: work: 398-3456

home: 495-2070

Name: Emilia Antecka

Phone No: work: 398-7192 ext 00384

home:

6. PROCEDURES & PROTOCOLS: Briefly describe

i) the biohazardous material involved (e.g. bacteria, viruses, human tissues) & designated biosafety risk group

We utilize human uveal melanoma cell lines in culture, they are designated biosafety risk 2.

ii) the procedures involving biohazards

Cell Culture