Characterization of Ocular and Metastatic Uveal Melanoma in an Animal Model

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PURPOSE. To characterize, in detail, tumor development, malignant cell dissemination, and metastasis in a 10-week animal model of uveal melanoma.

METHODS. One million 92.1 human primary uveal melanoma cells were injected into the suprachoroidal space of the right eye of 27 immunosuppressed albino rabbits. Intraocular tumor growth was monitored weekly by fundoscopy and by ultrasonography at the end of the experiment. To document the progression of the disease, one animal per week was killed. The enucleated eyes, lungs, and livers were macroscopically examined and histopathologically studied by hematoxylin and eosin, periodic acid-Schiff, and immunohistochimistry. Mononuclear layers isolated from the rabbits’ blood samples were cultured.

RESULTS. Histopathology showed intraocular tumors in 89% of the animals. Tumor growth was found 1 week after cell inoculation, and by the end of the experiment large tumor masses were observed. Microscopic pulmonary metastatic foci were first observed 4 weeks after cell injection. By the end of the experiment, all the animals had metastasis to the lungs.Interestingly, 18% of the animals also had micrometastasis to the liver. Viable adherent uveal melanoma cells were successfully isolated from peripheral blood and grown in vitro.

CONCLUSIONS. In this study, most rabbits developed intraocular tumors followed by lung metastasis, and some of these rabbits later developed liver micrometastases. This novel source of research material warrants a follow-up longer than 10 weeks to assess early, or treat metastasis. Animal models of uveal melanoma have been critical in advancing our understanding of the disease. 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 because tumors that develop in their natural microenvironment should present biologic and metastatic properties similar to those of tumors that develop in the clinical setting.

Animal models of uveal melanoma have been developed using melanoma cells derived from three different origins: mouse (B16),6 hamster (Greene),7 and human (ie, OCM,8 MKT-BR,9 SP 6.5,9 and 92.110). The mouse B16 melanoma cell line was primarily used in its natural host for the study of metastasis and the immunologic response to the disease.11-12 The hamster Greene melanoma cell line was broadly used to conduct preclinical research in rabbits.13-18 Both the Greene and the B16 melanoma cell lines present the disadvantages of being not only of animal origin but also of cutaneous origin. As a result, the biologic behavior of the tumors generated by these two cell lines might differ to some extent from that observed with human uveal melanoma cells.5,19

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 mice20 and immunosuppressed rabbits.4 The main advantage for the use of rabbits is their larger eye size, which permits a more accurate injection of the cell suspension in the posterior compartment and allows the use of conventional diagnostictic techniques for tumor follow-up. Since its establishment in 1989 by Kan-Mitchell and coworkers,8 the model has been successfully used in a variety of studies,21-22 primarily related to the primary tumor. However, the time-course of the progression of uveal melanoma after the inoculation of human uveal melanoma cells into the rabbit eye has not yet been described in detail. Understanding the progression of uveal melanoma in this model is crucial for the design of suitable preclinical studies intended to prevent or reduce metastatic burden. Only a thorough characterization of the model will allow for the correct interpretation of the changes produced.

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by new therapies. The aim of this study is to characterize in detail the processes of tumor development, malignant cell dissemination, and metastasis in a 10-week rabbit model of uveal melanoma.

METHODS

The animal model was carried out in compliance with the Association of Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The approval of both the Animal Care Committee and the Ethics Subcommittee at McGill University was obtained before all experiments were performed.

Animals

Twenty-seven male New Zealand albino rabbits (Charles River Canada, St-Constant, Québec, Canada) with a mean initial weight of 3.5 ± 0.2 kg (range, 2.9–3.6 kg) were used in this study. The animals were immunosuppressed using daily intramuscular injections of cyclosporin A (CsA; Sandimmune 50 mg/mL; Novartis Pharmaceuticals Canada Inc., Dorval, Québec, Canada) to avoid cell rejection. CsA administration was maintained throughout the 10-week experiment to prevent spontaneous tumor regression. The dosage schedule recommended in previous studies was used, as follows: 15 mg/kg per day for 3 days before cell inoculation and for 4 weeks thereafter, followed by 10 mg/kg per day for the last 6 weeks of the experiment. CsA doses were adjusted weekly according to each animal’s weight.

Cell Line and Cell Injection Procedure

The 92.1 primary human uveal melanoma cell line, kindly provided by Antonia Saornil from the Instituto Universitario de Ofthalmobiología Apicula, University of Valladolid (Valladolid, Spain), was used. This selection was based on previous studies performed in our laboratory in which these cells showed high proliferative and invasive potential in vitro.

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). They were then cultured in RPMI 1640 medium (Invitrogen, Burlington, Ontario, Canada), supplemented with 5% heat-inactivated fetal bovine serum (FBS; Invitrogen), 1% fungizone (Invitrogen), and 1% penicillin-streptomycin (Invitrogen). One million cells (cellular viability greater than 98%) were used as anesthetics during the surgical procedure.

Follow-up

Cyclosporin A Toxicity. During the 10-week experiment, the animals were monitored daily for signs of CsA toxicity, including regurgitation, diarrhea, and weight loss.

Fundoscopy. Indirect ophthalmoscopy of dilated pupils (Mydfrin; Alcon Canada Inc., Mississauga, Canada and Mydriacyl; Alcon Canada Inc.) was performed before cell inoculation to rule out any ocular abnormality and then 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).

Ultrasound Examination. Ultrasound examination using a 10-MHz B-scan and standardized A-scan (I 3 System-ABD; Innovative Imaging Inc., Sacramento, CA) was performed in 15 animals under anesthesia during the last week of the experiment to determine the echographic characteristics, location, and final dimensions of the tumors in vivo.

RESULTS

Euthanization. To document the time-course of the disease, particularly the development of metastasis, one animal was humanely killed per week. Selection was based on the appearance of the animal and signs of CsA toxicity. Remaining rabbits were humanely killed at the end of the experiment. The rabbits were euthanized by exsanguination after anesthesia using intramuscular ketamine (35 mg/kg)/xylazine (5 mg/kg). An autopsy was performed on every animal that was killed. Enucleated eyes and the organs with possible metastatic disease, such as lungs, livers, and kidneys, were collected, macroscopically examined, and preserved in 10% phosphate-buffered formalin.

Histopathologic Studies. Formalin-fixed, paraffin-embedded sections of the collected specimens were hematoxylin and eosin (H&E) stained for histopathologic assessment. Immunohistochemistry using the HMB-45 monoclonal antibody (mAb; DakoCytomation, Mississauga, Ontario, Canada) was performed according to the streptavidin-biotin complex (ABC) method. Microwave citrate treatment (citrate buffer pH 6.0) was used as the heat antigen retrieval technique.

Uveal melanoma histopathologic prognostic factors were evaluated weekly in the intraocular tumors of the killed rabbits throughout the course of the experiment and in 10 animals at the end of the experiment. Prognostic factors evaluated included cell type, tumor size, tumor infiltrating lymphocytes (TILs), vascular loops, mitotic rate, cytomorphometry (mean of the ten largest nucleoli), and extraocular extension. Tumors were classified into either the “high lymphocytic” group or the “low lymphocytic” group according to the number of TILs (100 or more and <100, respectively) per 20 high-powered (100×) fields (HPFs), as previously described. Eye sections were stained with periodic acid Schiff (PAS) to evaluate the presence of vascular loops. Mitotic activity was measured as the mean number of mitoses seen per 10 HPFs. The mean of the ten largest nucleoli (MTNL) in 25 HPFs was measured.

Blood Sample Processing. Blood samples were obtained by cardiac puncture at the moment of euthanization starting 6 weeks after cell inoculation. The presence of CMCs was assessed by culturing the mononuclear layer isolated from whole blood samples using the Ficol-Paque Plus method (Amersham Biosciences, Baie d’Urfé, Québec, Canada). Mononuclear layers from 2 mL blood samples from 19 rabbits were cultured in RPMI 1640 media supplemented with 10% FBS at 37°C. Subsequently, uveal melanoma cells were identified through specific immunostaining of cytoptins or cells grown in coverslips using HMB-45, melan-A (Novocastra Laboratories Ltd., Burlington, Ontario, Canada), and tyrosinase (Novocastra Laboratories Ltd.) mAbs; 92.1 cells served as a positive control.

Survival Rate and Animal General Condition

The survival rate in this animal model was 90%. Because one animal was killed per week, 19 of the initial 27 animals were supposed to survive until the 10th week. Seventeen rabbits survived until the end of the experiment. Only 2 rabbits had to be killed before planned, the first one during the first week and the other in week 8, each because of acute weight loss.

The animals remained in good condition throughout the experiment and had only minor signs of CsA toxicity including occasional diarrhea or, in some cases, regurgitation. One of the major side effects of CsA is weight loss. We observed an initial acute weight loss 1 week after cell injection. After week 1, the animals maintained their weight until the end of the experiment, indicating that CsA doses were well adjusted (Fig. 1).

Intraocular Tumor Studies

Fundoscopy. During the 10-week experiment, fundoscopy allowed for the detection of intraocular tumors in 67% of the animals (n = 27; Fig. 2). Some tumors (7 of 25) were readily detectable 2 weeks after cell injection. Others became detect-
able later. 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 (Fig. 3).

**Ultrasound Examination.** Using A-scan, intraocular tumors were observed as solid lesions with low to medium regular internal reflectivity. Using B-scan, intraocular tumors were observed as large choroidal masses (Fig. 4). Ultrasound detected the presence of intraocular tumors in all 15 animals examined. Ciliary body involvement was detected in 4 of the 15 (27%) animals studied. Extraocular extension was observed in 9 of the 15 animals (60%). Ten weeks after cell inoculation, tumors measured 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 animals, 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.

**Gross and Histopathologic Examination.** Enucleated eyes were examined postmortem to determine the presence and morphology of the developed intraocular tumors. Macroscopically, intraocular tumors were found in 82% of the animals (22 of 27) (Fig. 5). Tumor development was observed in the first killed animal 1 week after cell injection. Histopathologic evaluation of the enucleated eyes further revealed that tumors were present in 89% of the animals (24 of 27). Mela

**Histopathologic Prognostic Factors**

Evaluation of uveal melanoma histopathologic prognostic factors reflected the high aggressiveness of the ocular tumors (Fig. 7). All tumors were of a mixed cell type, with predominantly epithelioid cells (80% of the tumor field). Marked lymphocytic in 82% of the animals (22 of 27) (Fig. 5). Tumor development was observed in the first killed animal 1 week after cell injection. Histopathologic evaluation of the enucleated eyes further revealed that tumors were present in 89% of the animals (24 of 27). Melanoma tumors were positively immunostained with HMB-45 mAb.

Intraocular tumor development was monitored throughout the experiment (Fig. 6). Tumors grew diffusely within the choroid during the first 2 weeks. The site of cell injection could be identified as a thickened choroidal area. By weeks 3 and 4, tumors began to grow in height, adopting a nodular or dome-shaped configuration. From week 5 on, the enucleated eyes had large tumors that varied in shape. By weeks 8 and 9, a considerable part of the tumor filled the vitreous cavity in all positive cases. At the end of the experiment, tumors completely filled the globe in 30% of the animals. In those large tumors, areas of necrosis were observed.
infiltration was found within the melanoma masses. With the exception of tumors from week 1, which showed <100 TILs per 20 HPFs, all tumors had >100 TILs per 20 HPF, and they were classified as belonging to the high lymphocytic group. From week 2 on, all tumors exhibited vascular loops. The mean number of mitotic figures seen in 10 HPFs was 48.7 ± 13.9 (n = 16). A significantly lower value (30.5) was obtained for week 1 tumors. The MTLN1 measured in 25 HPFs 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 after week 4. At the end of the experiment, extrascleral extension was observed in 90% of the animals.

**Malignant Cell Dissemination**

We successfully isolated CMCs from blood samples obtained at the moment of euthanatization. Colonies, usually 2 or 3 per sample, were observed 2 weeks after seeding of the mononuclear layers. Cells were positively identified as uveal melanoma cells through immunostaining using HMB-45, melan-A, and tyrosinase mAbs (Fig. 8). In vitro proliferating CMCs were first isolated 6 weeks after cell inoculation, when we first processed blood samples. CMCs were detected in 5 of 19 (26%) cultured blood samples. Examination of cultured CMCs grown on coverslips revealed that the cellular morphology resembled that of the original 92.1 human uveal melanoma cell line.

**Metastasis**

Gross examination of the lungs revealed multiple nodular lesions compatible with metastasis 7 weeks after surgery (Fig. 9). Lung metastases were macroscopically observed in 41% of the animals remaining at the end of the experiment. 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 showed histopathologically confirmed lung metastasis (Fig. 9). Metastatic lesions were HMB-45 positive (Fig. 9). The lesions usually presented as multiple subpleural micronodules surrounded by a peripheral rim of lymphocytes. Interestingly, micronodular metastatic lesions were found in the livers of 3 of 17 (17.6%) animals remaining at the end of the 10th week (Fig. 9).
DISCUSSION

Detailed characterization of uveal melanoma progression, including tumor development, malignant cell dissemination, and metastasis, was undertaken using the albino rabbit model. By using the strategy of killing one animal per week, we were able to demonstrate histopathologically that the disease progresses in a sequential manner.

Intraocular tumors successfully developed in most animals. Tumors grew inside the eye during the first 5 weeks and extended extraocularly thereafter. They displayed similarities to human uveal melanoma in cytoarchitecture, morphology, phenotype, and microcirculation patterns. Tumors in this animal model were, however, more aggressive and highly vascularized and were composed predominantly of epithelioid cells with high mitotic activity and MTLN1. A highly aggressive human uveal melanoma cell line was selected on purpose to model in less than 3 months a disease that can take 10 years or more to metastasize in humans. Consequently, the disease progressed and spread over a shorter period, a desirable feature in an animal model with metastatic disease.

Tumor presence and growth were observed in vivo by fundoscopy and ultrasonography. Intraocular tumors could not be detected by fundoscopy in 22% of the animals, either because the animals had severe intraocular hemorrhage or retinal detachment or because the tumors were not clinically visible. In those animals, postmortem pathologic examination demonstrated the presence of a tumor. Alternatively, ultrasound was particularly useful to overcome the limitations of fundoscopy, allowing for a more accurate determination of tumor location, dimensions, and extraocular extension (Blanco PL, et al. IOVS 2003:44; ARVO E-Abstract 3649).

To the best of our knowledge, this is the first time that CMCs were isolated and cultured from peripheral blood in an animal model of uveal melanoma. We demonstrated that these cells were indeed viable and that they do maintain the expression of specific melanoma markers. Our first attempt at isolation started in week 6, leading to successful cultures. Given that the CMC lifespan in the peripheral blood is known to be short,29 we can rule out the possibility that these isolated cells were mechanically injected into the circulation at the time of initial choroidal injection. In addition, another animal model was recently conducted in our laboratory following the same protocol, and no viable CMCs could be isolated in any animal immediately after surgery or at week 1. In view of cancer as a clonal proliferation of cells, we can postulate that these cells are in fact released from the primary tumor shortly before the time of isolation, initiating the first steps of the metastatic cascade. The final role of CMCs in uveal melanoma is yet to be determined.30–32

Several studies reported in the literature have demonstrated the value of detecting CMCs in the bloodstream of patients using RT-PCR.33–36 Although PCR has been proven to be highly sensitive and specific, only by culturing can viable CMCs be identified. Because only viable cells have the potential to metastasize, the strategy of culturing will indicate a more realistic risk for metastasis development. The availability of cultured CMCs opens a new avenue for the use of in vitro techniques that can provide thorough characterization of these cells. In addition, using genomic profiling technology, we can compare CMCs with primary tumor cells and metastatic cells to elucidate the unique changes that malignant cells undergo as they escape the eye. Ideally, both methods—RT-PCR and cell culture—should be used to take advantage of their individual strengths.

Little progress has been made in the study and treatment of metastatic uveal melanoma, partly because of the lack of a suitable animal model. Our experimental model exhibited a high metastatic rate, a desirable feature for the evaluation of new therapeutic strategies for metastatic uveal melanoma, not previously reported in similar animal models.7 The high rate of
metastasis achieved enables the study of systemic tumor dissemination. In addition, the effect of therapies targeted against metastasis can be explored. Interestingly, the response to treatment can be assessed not only histopathologically at the end of the experiment but also dynamically during the experiment, observing the effect of therapy in the presence of CMCs.

In our animal model, the lung was the primary site for metastasis. Hepatic micrometastases were also found to a lesser extent. This finding is particularly appealing because liver metastases have not yet been reported using this particular experimental model or similar ones and, most important, because the liver is the preferential site of metastasis in human beings. The ability of these human uveal melanoma cells to metastasize first to lung and then to liver in the rabbit remains intriguing. This animal model may provide a unique opportunity to test factors involved in tumor homing and to examine the role that the organ microenvironment plays in the development of metastasis. Extending the length of the experiment may result in a higher rate of liver metastasis. We believe that the experiment can be prolonged because low mortality rates were observed and the animals reached the end of the experiment in good condition.

One of the main limitations of this animal model is the need for continuous immunosuppression to allow for efficient tumor development. The use of CsA can result in severe toxicity that may limit the length of the experiment and thus the time in which metastatic disease can fully develop. Moreover, short-term experiments may limit the time in which therapeutically effective effects can be demonstrated when using the model in preclinical trials. However, careful selection of the initial animal weight (~3 kg) combined with strict dosage regimen and efficient animal care in this study resulted in minimal CsA side effects. As a consequence, many of the animals were able to survive the entire length of the experiment, ensuring a high metastatic rate. The selection of the weakest animal for the weekly euthanatization might also have influenced the overall survival rate.

In conclusion, this study has advanced our understanding of the progression of uveal melanoma in this animal model. It also provides baseline information for future experiments, particularly those aiming to develop new diagnostic or therapeutic approaches not only for the primary tumor but also for the metastatic disease. For instance, we can test the impact that early primary tumor therapy has in the presence of CMC and consequently in the prevention or reduction of the metastatic burden. In addition, the model offers great potential to further characterize CMC dissemination. Success in the isolation and culture of CMCs encourages future cultures of CMCs from patients with primary tumors, increasing our understanding of this subclinical stage of metastasis. This model suggests that culturing CMCs from patients with primary tumors might be a useful way to identify patients at high risk for metastatic disease or even patients with subclinical metastases who could benefit from early systemic therapy. We are working on these lines of research. Progress in these areas will ultimately be translated into clinical benefit for patients affected by uveal melanoma.

References

27. McLean IW, Keefe KS, Burnier MN. Uveal melanoma: comparison of the prognostic value of fibrovascular loops, mean of the ten


