Whole Body Bioluminescent Imaging of Human Uveal Melanoma in a New Mouse Model for Local Tumor Growth and Metastasis

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Abstract

Purpose: Human uveal melanoma develops in one of the most capillary-rich tissues of the body and has a pure hematogenous dissemination. Radiodiagnostic examinations, such as ultrasonic diagnostic resonance imaging and chest radiographs plus liver enzyme studies in blood, are methods to detect liver and other distant metastases in patients. Nevertheless, the mortality rate is high, because of the frequent occurrence of metastases and the lack of systemic therapy. Therefore, the development of novel anticancer strategies is urgent, and more sensitive and less invasive methods of detecting and monitoring in vivo tumor growth and metastatic disease in cancer models are needed.

Methods: A luciferase (lac)-positive human uveal melanoma cell line (OCM-1 FRT/luc) was established. Tumor cells were inoculated into the anterior chamber of murine eyes for induction of orthotopic growth or into the left heart ventricle to mimic hematogenous micrometastatic spread. Development of metastases and tumor growth was monitored weekly by whole-body bioluminescent reporter imaging (BRI).

Results: Injection of cancer cells into the anterior chamber of the eye of mice closely mimicked orthotopic tumor growth of uveal melanoma. Tumor progression could be quantitatively monitored 3 weeks after inoculation of $10^5$ OCM-1 FRT/luc cells. Of the mice injected, 83% exhibited a detectable tumor within 5 weeks. Intracardiac injection of tumor cells resulted in metastatic growth, especially in bone. Mice had bone (maxillofacial region and femora) and visceral (lung and mediastium) metastases after 4 to 6 weeks. OCM-1 FRT/luc cells may also have a propensity to colonize the eye after intracardiac inoculation.

Conclusions: BLI enables continuous quantitative monitoring in the same animal of growth kinetics for each tumor and its metastases. This model will accelerate the understanding of the pathogenesis and treatment of uveal melanoma and metastasis.
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Introduction

Uveal melanoma develops in one of the most capillary-rich tissues of the body and has pure hematogenous dissemination. The mortality rate is high, because of the frequent occurrence of metastases, mainly in the liver (87%), lungs (46%) and bone (29%) \(^1\). Early detection is important, because early treatment of the primary tumor and metastases is linked to patient’s survival \(^2\).

Liver and other metastases are an early or a late event in the evolution of metastasis and can be detected by liver function analysis, ultrasound scan of the liver, or postmortem examination \(^3\). There is no evidence that early detection of micrometastasis has led to improved survival. It is important to note that formation of micrometastasis is the pathologic basis for the occurrence of clinically overt metastasis. Therefore, therapeutic strategies designed to interfere with initial events in organ colonization and metastasis are needed. Current in vivo models \(^4, 5, 6\) lack the sensitivity that is necessary to detect the initial processes, such as the angiogenic switch \(^7\), essential for tumor progression. Furthermore, disturbed levels of liver enzymes in blood are an indirect measure of tumor burden. More sensitive methods of detecting and monitoring tumor and metastatic growth directly are needed. A commonly used animal model for induction of tumor growth involves orthotopic inoculation of human uveal melanoma cell lines into the anterior chamber of the eye of immunodeficient (nu/nu) mice \(^4, 5\). Intracardiac injection of tumor cells in immunodeficient mice can be used as a model of experimental metastasis \(^8, 9\).

Expression in vivo of reporter genes encoding bioluminescent or fluorescent proteins can be detected externally by sensitive detection systems \(^10\). Cancer cell lines stably transfected either with the firefly luciferase (luc) or the green fluorescent protein (GFP) have been used to monitor local tumor growth and metastasis in living mice \(^11, 12, 13\).

We investigated the possibility of whole body bioluminescent reporter imaging (BLI) to visualize in vivo the development of local tumor growth and metastasis after inoculation of luciferase-transfected OCM-1 cells (OCM-1 FRT/luc) \(^4\), a human uveal melanoma cell line, into the anterior chamber of the eye (orthotopic site) and to study experimental metastasis in immunodeficient (Balb C nu/nu) mice in vivo \(^8, 14\).

In this study, BLI allowed sensitive, noninvasive, and quantitative localization and monitoring of orthotopic and metastatic growth of uveal melanoma in vivo.

Materials and methods

Animals

Female Balb C nu/nu mice were purchased from Charles River (Charles River, The Netherlands). They were housed individually in ventilated cages under sterile conditions according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Sterile food and water were provided at libitum. Mice were 8 weeks old at time of intraocular
or intracardial injection of tumor cells. For surgical manipulation, mice were anesthetized with an intraperitoneal injection of a mixture of ketamine hydrochloride (0.66 mg/kg body weight; Eurovet) and xylazine (0.13 mg/kg body weight; Bayer) in PBS. Mice were killed by CO₂ at first signs of distress or after a 12-week observation period.

Figure 1: Psv40 is the promoter, sv40 PA is the stop vector, Amp is ampicilnine, and Pcmv is the CMV promoter (A) Transfection of first construct (Flp-In) with resistance for blasticidine. Stable blasticidine-resistant clones were isolated, subcloned and tested for their presence of FRT cassette; (B) Cotransfection was performed between the FRT+ tumor clone E11 with the Flp-In expression vector pcDNA5/FRT and the Flp recombinase expression vector pOG44. The gene of interest (GOI) was integrated into the FRT site using 1μg construct CMV-luciferase FRT; (C) Final result of construct after the recombinase by pOG 44 with resistance for hygromycin and FuGENE-6. Stable transfectants were selected with hygromicine (400μg/ml, Invitrogen).
Human uveal melanoma cell line
OCM-1 cells were obtained from primary uveal melanoma. They grew as monolayer in 10 ml/dish Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% FCS (Hyclone), 100 IU/ml penicillin (Gibco) and 100 µg/ml streptomycin (Gibco). Cell cultures were incubated at 37°C in a humidified atmosphere and a CO₂ content of 5% in air.

Establishment of stable transfectants expressing the luciferase reporter gene:
OCM-1 cells were transfected with 1µg Flp-in construct (figure 1, Invitrogen) using FuGENE-6 according to the manufacturer’s protocol (Roche Biochemicals, Almere, The Netherlands). For better selection of stable transfectants, the Zeocin resistance gene (original vector) was replaced by blasticidin-resistance gene (blasticidin, 3.0 µg/ml, Invitrogen). Stable blasticidin-resistant cell clones were isolated, subcloned, and tested for the presence of a single FRT cassette by Southern blotting analysis. Only those clones containing one Flp construct in their genomic DNA were used. Subsequently, a FRT-positive clone (E11) from OCM-1 was used for the generation of a luciferase-expressing cell line. Cotransfection was performed between the FRT+ tumor clone E11 with the Flp-In expression vector pCMV6/FRT and the Flp recombinase expression vector pOG44. The gene of interest (GOI) was integrated into the FRT site, by using 1µg construct CMV-luciferase FRT (figure 1, firefly luciferase cDNA) and FuGENE-6 (Roche Biochemicals, Almere, The Netherlands). Stable transfectants were selected with hygromicine (400 µg/ml, Invitrogen). Subsequently, the OCM-1 E11 FRT/luc was tested for expression of luciferase activity per cell (figure 2). The clone was cultured up to passage 15. Expression of luciferase in OCM-1 FRT/luc cells remained stable over more than 10 passages. This luciferase expression OCM-1 clone was therefore used for in vivo evaluation by BLI.

Figure 2: Luciferase activity of OCM-1 FRT/luc cells correlated in a linear fashion with the cell number ($r^2=1.0$). Values are expressed as relative light unit (RLU) and number of cells per 0.5 ml.
The murine model of human uveal melanoma in the anterior chamber of the eye

Tumor cells were inoculated into the anterior chamber of mouse eyes (only right eyes, n = 12) as described earlier by Niederkorn. With a glass needle OCM-1 FRT/luc cells at a concentration of $10^5/4\mu l$ were injected into the anterior chamber of the eye of 8-week-old female Balb C nu/nu mice. After 4 to 6 weeks, eyes with large tumors were enucleated and used for immunohistochemistry. After 12 weeks, all mice were sacrificed. Mice were imaged for the first time after 1 week with BLI and followed weekly.

Induction of metastasis by intracardiac injection of cancer cells

A single cell suspension of OCM-1FRT/luc cells ($1\times10^5/100\mu l$ PBS) was inoculated into the left heart ventricle (n= 7) according to the method described by Arguello and co-workers with modifications. Development of metastases was monitored weekly by BLI. During the entire experimental period of intracardiacally inoculated mice, none of the metastases detected by BLI showed a decline in bioluminescent activity. After 12 weeks all mice were killed and metastases were dissected for immunochemistry.

Bioluminescent reporter imaging

We used an imaging unit, (IVIS Imaging System 100, Xenogen), that consisted of an intensified 25-mm square, back-thinned, back-illuminated, charge-coupled device camera fitted to a light-tight chamber and equipped with a f0.95/16,5 mm lens. The imaging system is managed by analysis software (Living Image; Version 2.20; Xenogen Corporation, Alameda, CA).

For detection of luciferase-expressing cells, mice were anesthetized as described earlier. Thirty microliters of a 250-mM aqueous solution of luciferine (D-luciferine Na salt; Molecular Probes, Leiden, the Netherlands) was injected intraperitoneally before photon recording began. Mice were placed in the light-tight chamber. Photon emission was then integrated for 20 seconds and creating pseudo-images. The bioluminescent signal was quantified by measuring the amount of highlighted pixels in the area shaped around each site of photon emission with the aid of the Living Image software (Version 2.20, Xenogen Corporation, Alameda, CA).

Histochemistry

After enucleation, eyes were immediately fixed in 5 ml of 4% para formaldehyde (Lommerse Pharma, the Netherlands). After 24 hours, the eyes were dehydrated for 2 hours in ethanol 70%, 1 hour in 90% and 30 minutes in 99% and processed for paraffin embedding. Goldner Trichome (to demonstrate the presence and maturation of newly formed bone), hematoxylin-eosin (H&E) and Periodic acid-Schiff (PAS) staining were performed on 5-µm serial sections of tumorous eyes and metastases. Bone metastases were selectively excised and fixed overnight in fixative at 4°C. After decalcification in EDTA acid they were processed for
Results

In vivo monitoring of orthotopic tumor growth
Of the mice injected with the tumor cells, 83% exhibited a detectable tumor within 5 weeks after intraocular inoculation of OCM-1 FRT/luc cancer cells, as detected by BLI. The first evidence of bioluminescent emission from orthotopically implanted uveal melanoma cells, (OCM-1 FRT/luc), was registered between days 21 and 37 after injection into the anterior chamber of the eye. The light emission in the tumorous eye increased substantially from first appearance until the day of enucleation (figure 3). Quantification of the luciferase signal, localized in the eyes with tumor burden from the time of their first appearance to their time of enucleation, was used for in vivo monitoring of the tumor growth (figure 3). Histological analysis (H&E and Goldner staining) confirmed the presence of cancer cells in the eye to be the site of bioluminescent emission by BLI (figure 4 and 6). The tumor burden correlated with bioluminescent signal (figure 4). The total volume of the smallest detectable tumor burden was 0.5 mm³.

In vivo monitoring of the development of distant metastases after intracardiac injection of cancer cells
After intracardiac inoculation of 10⁵ OCM-1 FRT/luc cells, four of the seven mice had bone metastases in the maxillofacial region after 4 weeks, detected by BLI (table 1, figure 5). Some mice also had metastatic tumor growth in the hind leg (three of seven), in the eye (two of seven) or in the diaphragm after 4-6 weeks (table, figure 5). The first luciferase signal of metastasis was registered at day 35 after intracardiac injection of cancer cells. In 57% (n=7) of the mice, the luciferase signal was localized in the maxillofacial region (figure 5). The intensity of light emission at the metastatic site increased substantially from the first day of appearance until the last day of the experiment. Additional metastatic sites became apparent by BLI at day 42 (figure 5). The quantification of the luciferase signal, localized over the different metastatic sites, allowed continuous monitoring in vivo of the tumor growth for each separate metastatic lesion from the time of appearance until the time of death. Histochemical analysis (H&E, Goldner and PAS + staining) confirmed the presence of cancer cells in metastatic lesions at sites of bioluminescent emission.

Highly invasive and metastatic uveal melanoma cells are capable of generating PAS-positive loops in vitro. We found PAS-positive loops and networks in all metastatic lesions that were induced by OCM-1 FRT/luc cells (figure 6).
Figure 3: BLI monitoring of OCM-1 FRT/luc cells injected into the anterior chamber of the eye. Bioluminescent photon emission was externally imaged from the lateral projection of the same mouse (A) 37, (B) 44, (C) 51 and (D) 58 days after injection. Signals are displayed as pseudocolor image (blue = least intense, white = most intense). (E) Growth curve obtained by quantification of the bioluminescent signal localized in the right eye in the same mouse. (F) Growth curves obtained by quantification of the bioluminescent signal localized in the right eye in six mice.

Discussion

This investigation presents for the first time intraocular and systemic inoculation with luciferase-positive uveal melanoma cells as a new model for monitoring orthotopic tumor growth and metastatic sites. Injection of OCM-1 FRT/luc cells in the anterior chamber of the eye and the model for induction of experimental metastases of the same cells in nude mice combined with BLI resulted in early detection and continuous monitoring of tumor growth in vivo.

Several groups have used a model of inoculation with uveal melanoma cells in which histological staining of tumor sections and liver enzyme tests were used to analyze therapeutic results.
Figure 4: Tumor burden after injecting tumor cells in the eye correlated in a linear fashion with bioluminescent signal of the same eye before enucleation ($r^2=0.996$). H&E staining of four different eyes at various stages during tumor progression.

Figure 5: BLI monitoring of OCM-1 FRT/luc cells injected into left cardiac ventricle. Bioluminescent photon emission was externally imaged from the frontal projection of the same mouse (B) 36, (C) 43, (D) 50 days after injection. Growth curve obtained by quantification in the same mice of the different bioluminescent signals localized at several metastatic sites (A).
Figure 6: Histological identification of OCM-1 FRT/luc cells in the orthotopic tumor and in distant metastasis. Hematoxylin-eosin, Goldner and PAS + staining of sections of tumor growing in the eye (A magnification: 4x, 40x; B magnification: 10x, 20x), in maxillofacial region (C magnification: 10x, 20x; D magnification: 10x, 20x) and a distant metastasis (E magnification: 10x, 20x) T = tumor, C = cornea, B = Bone, arrows = PAS + loops.
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**Table:** Overview of the metastatic sites after intracardiac injection of OCM-1 FRT/luc cells in mice

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*Liver, diaphragm; Subcutaneous, mediastinum*

These in vivo models, with *in vivo/ex vivo* methods, have several limitations. First, several groups of animals must be killed at different time points of an experiment to retrieve temporal information. Second, positive blood samples are a late event in metastatic disease. Third, monitoring of tumor growth in the anterior chamber can be difficult when edema develops in the cornea or a tumor starts to grow in the posterior segment of the eye. For these reasons, BLI is time saving and results in generation of more data per experimental series, leading to statistically sound results obtained more rapidly and with the use of fewer numbers of animals.

BLI provides an opportunity to detect a small numbers of malignant cells rapidly, over the entire body of the mouse. The high sensitivity and specificity for detection of cells in all compartments of an intact animal frequently uncovers biological phenomena such as tumor escape mechanisms and patterns of tumor cell metastasis. This capability enables the study of metastatic and minimal residual disease states in animal models. In the present study, BLI of uveal melanoma cells in the anterior chamber of the eye allowed detailed anatomical information, continuous noninvasive sensitive monitoring and precise quantification of tumor growth in vivo.

Furthermore, micrometastases are difficult to detect because of the lack of clinical signs in mice. Induction of metastasis by intracardiac injection of uveal melanoma cells will provide the opportunity to explore preferential sites for metastasis. According to the literature, the OCM-1 cell line is capable to metastasize *in vivo*. In our study, after intracardiac injection, OCM-1 FRT/luc cell line induced metastases in maxillofacial region, the eye and hind legs. These findings support the concept that organ specificity of metastasis is not due to differences in blood perfusion, but primarily depends on local interactions between cancer cells and the organ-specific (micro)environment.

This OCM-1 FRT/luc model provides a valuable experimental setting to preclinical evaluation.
the in vivo antitumor activity of investigational agents in the same animal over time. Also responses at the different metastatic sites in the same animal can be easily tested. Many gene-profiling studies have been published during the last years, reporting different genes that can play an important role in the metastatic potential of uveal melanoma. Another advantage of this model is the opportunity to explore the pathogenic role of these different genes of interest by transfecting the OCM-1 FRT cells.

In conclusion, we have established a new model of intraocular and intracardiac injection of luciferase reporter positive transfected cancer cells. Luciferase positive uveal melanoma cells coupled to BLI detection allowed continuous noninvasive monitoring in vivo of tumor growth and metastatic growth. This model will facilitate studies of the molecular mechanisms involved in early stages of tumor development and the development of new therapies.

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