Review Article

Uveal Melanoma Metastasis Models

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Metastatic disease · Uveal melanoma · Metastasis models · Liver metastases

Abstract

Metastatic disease is the leading cause of death among patients with uveal melanoma. Treatment options for patients with clinically disseminated disease are usually unsuccessful. In vitro and in vivo models are important tools to investigate the pathogenesis of metastatic uveal melanomas and develop treatments for the metastases. In vitro experimental approaches focusing on cell invasion/migration which mimic the steps of the complex metastatic process may also be used for the identification of potential anti-invasion/migration drugs that may inhibit the spreading of tumor cells or the development of metastases. The effects of these drugs must subsequently be confirmed in reliable in vivo models before entering the clinical trial phase. Several models of intraocular melanoma with metastases in rodents and rabbits are currently being used. Most experimental models of uveal melanoma metastases require injection or implantation of melanoma cells into orthotopic locations, including into the liver, spleen, tail vein, or the left ventricle of the heart, in order to mimic the metastatic process.

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Introduction

Metastasis, the spread of cancer cells from a primary site and the formation of new tumors in another region of the body, is responsible for as much as 90% of cancer-associated mortality [1, 2]. The liver is the exclusive site of systemic metastasis in 40% of the patients and is often the first metastatic site in patients with uveal melanoma [3]. Despite advances in the diagnosis and treatment of the primary tumor, the 5-year mortality rate of uveal melanoma patients has not significantly changed over many years [4]. When metastases have developed, the patients' median survival is 5–7 months [5]. Liver metastases may occur years after the

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apparently successful treatment of the primary ocular tumor. The establishment and appropriate use of in vivo and in vitro metastasis model systems are useful for designing rational and effective therapeutic strategies.

In vitro Uveal Melanoma Assays Useful for Studying the Metastatic Process

The metastatic process involves a complex sequence of interrelated steps, which include neovascularization and increase in size of the primary tumor, detachment of neoplastic cells and entry into the circulation, adhesion to the endothelium of distant organs, passage through the capillary basement membrane, and proliferation to form a secondary tumor [6]. In vitro assays have been developed to mimic a part or parts of the metastatic cascade.

Invasion Assays

Invasion is considered a prerequisite for a metastatic phenotype of tumor cells, whereas cell locomotion and adhesion may be involved in the metastatic process [7, 8]. A migratory ability and the capacity to degrade the extracellular matrix by proteases are necessary for tumor cells to penetrate the extracellular matrix [9]. Certain in vitro culture systems may be used to evaluate the adhesion, invasion, motility, and growth of tumor cells as well as mimic the microenvironment of target organs by modulating culture conditions. Both invasion and chemotaxis of tumor cells can be determined by using transwell cell culture chambers. For an invasion assay, the transwell membranes are coated with matrigel, the tumor cell suspension is added to the upper compartment in a serum-free medium, and the lower compartment is filled with conditioned medium. After 24 h, the medium at the upper surface of the filter is removed and the transwell membrane is fixed. The noninvasive cells and the matrigel are then removed. Next, the transwell is washed, and the invasive cells on the lower surface of the filter are stained and counted under a microscope. A chemotaxis assay is conducted in the same manner except that the transwell membranes are not coated with matrigel, and certain chemoattractants, such as chemokines and extracts of specific organs, are added to the lower compartment [10]. Endothelial cell cytokines, matrix components, and degradation products are all chemotactic for tumor cells. The invasion level is thus defined as the percentage of cells passing through the filter. Digital image analysis of paraffin wax-embedded inserts can also be used to quantify the invasion [11].

Interactions of tumor cells with the vascular endothelium and basement membrane are crucial in these processes, involving tumor cell attachment to and penetration through the vessel wall and underlying matrix at both the primary and distant sites. The degradation of the basement membrane has also been correlated with the metastatic potential of tumor cells. The penetration of circulating tumor cells into the endothelium is a crucial step for tumor metastasis [12–19]. A modified in vitro invasion assay has been developed to assess uveal melanoma invasion across endothelial and basement membrane barriers [20]. The transendothelial cell invasion model adopted a modified Boyden chamber system as well as human dermal microvascular endothelial cells and microvascular endothelial cells freshly extracted from human liver resections; it was used to assess the invasion of cells grown from 13 different primary posterior uveal melanomas obtained at enucleation. Endothelial cells were grown to confluency on a polycarbonate membrane precoated with an artificial basement membrane. Tumor cells were added to the upper chamber, and RPMI-1640 medium with 0.1% BSA was added to the lower chamber; after incubation at 37°C for 24 h, noninvading cells were removed from the upper chamber by gently wiping the upper surface of the membrane. After

staining of the membranes, the level of invasion was assessed by counting the number of cells present in 10 fields on the lower surface of the membrane under a microscope. The presence of the endothelial cell layer reduced invasion in all primary uveal melanoma cultures, but the effect varied between tumors. This study showed an association of transendothelial cell invasion in vitro with known prognostic markers and, as such, may more reliably distinguish between melanomas invading at high and low levels than previous invasion assays using a basement membrane alone [20]. The transendothelial assay is too impractical to be of prognostic value in daily use. Long-term clinical follow-up is necessary to further correlate in vitro transendothelial invasion in this model with clinical outcomes; however, it is possible that this system may be a more representative model of invasion than previous in vitro models [20].

Migration Assays

Migration is an essential part of the formation of metastases and relies on the migratory activity of tumor cells at two phases of the metastatic process. First, the cells detach and emigrate from the primary tumor. Subsequently, they enter lymphatics or blood vessels and disseminate. Second, they extravasate from the vessels and re-enter the tissues at distant sites, where they develop metastases. Although the cells migrate autonomously, they are not independent of their microenvironment. Cell migration is a complex regulatory process composed of cell-cell and cell-matrix interactions as well as of influences of signal substances such as chemokines, cytokines, neurotransmitters, and hormones [9, 21]. The following methods can be used for the investigation of cell migration.

Wound Healing Model or Monolayer Wound Assay

The wound healing model or monolayer wound assay is also called the scratch wound assay and consists of scratching a confluent cell monolayer with a pipette tip. The monolayer recovers, which can take several hours or several days, depending on the type of cell, the conditions of the medium, and the extent of the wound. This process can be monitored either by manually imaging samples fixed at particular moments or by time-lapse microscopy. As a measurement of the rate of cell locomotion, cell migration is usually evaluated by determining the rate of advance of the wound edge or, more generally, the areas recolonized by the cells by counting the number of migrating cells, that is, the number of cells observed across the wound borders [22–26].

Microcarrier Bead Assay

Cells are grown to confluency on microcarrier beads. Cell-coated beads are placed in 24-well plates, where they may be treated or untreated. After a period of time, the beads are removed by suction or washes, and the cells that have migrated from the beads to the plastic surface of the well are fixed and stained with crystal violet. The number of the migrated cells is then evaluated either by visual inspection or by reading the associated optical density [27, 28].

Ring Assay

Cancer cells are initially confined to the circular central region of a Teflon or glass ring placed in a 6-well culture plate. After several hours of culture to ensure a confluent layer in the enclosed area, the ring is removed, the culture is rinsed to remove unattached cells, and the remaining cells are allowed to migrate over the surface for a number of hours. The cells are then rinsed, fixed, and stained with toluidine blue. Cell migration is measured either as

the net increase in the total area covered by the cells or as the average of the linear distances covered by the migrating cells, such as the maximum distance from their points of origin. This quantification is usually based on digitized images of the stained cultures submitted to the software able to measure surface areas or linear distances [29–31]. Another assay based on similar principles is the aggregate migration assay, where cell aggregates are prepared by centrifugation and sedimentation and plated onto a coated glass slide to enable them to migrate over its surface. This assay was initially designed to study the migration of cells from freshly excised human tumors and was later adapted to cell lines [32].

Single-Cell Tracking and Cell Chemotaxis Assay

Single-cell tracking consists of automatically recording sequences of frames of living cells in standard cultures by means of microscope video acquisition systems. The location of each cell is tracked over sequences to establish the cell trajectories from which qualitative and quantitative features are obtained that characterize the cellular dynamics, such as distance, speed, direction, and the duration of the migration. Manual or interactive computer-assisted tracking has been used [33–36]. The cell chemotaxis assay is based on the principles of single-cell tracking for an efficient way to study cell chemotaxis and requires direct-viewing chambers enabling easy image acquisition and a stable and linear chemical gradient to be maintained for a sufficient period of time. Devices such as the Dunn chamber designed by Dunn and colleagues [37] have been used in a number of chemotaxis studies [38–40]. The Dunn chamber takes the form of 2 concentric wells separated by an annular platform 20 μm below the top of the chamber. When the inner well is filled with a control medium and the outer with a medium containing a potential chemoattractant, a radially directed linear diffusion gradient develops. Cells are cultured on a coverslip that is inverted onto the Dunn chamber, and the cells that migrate out of the annular platform can be recorded under a phase-contrast microscope using automatic recording. Individual cell trajectories can be established on the acquired images, and each trajectory can be characterized by a directional vector and angle.

Colloidal Gold Migration Assay

This single-cell migration assay is one of the first techniques used for the visualization of the individual tracks of cultured cells moving on a 2-dimensional substrate [41]. It consists of culturing cells on a glass coverslip covered with gold particles. After a period of time, the cells are fixed and the coverslips mounted onto glass microscope slides. This cell migration computer-aided analysis may be used to monitor the images of the tracks made by the cells and the distances that they migrated [42].

Summary of Migration Assays

To quantify the anti-invasion/migratory effects of drugs, experiments using the ring assay and wound model are used. The colonization ability involves the following two components: migration and growth; these should thus both be taken into account when interpreting the results [43, 44]. Indeed, experimental conditions often have an impact on both proliferative and motile activities, with the latter depending on the proportion of motile cells as well as on intrinsic migration parameters (e.g. cell speed, cell direction, persistence, duration, etc.), requiring the establishment of single-cell paths to be measured. The wound healing model is particularly well adapted for applications in relation to tissue injury, such as testing cell responses and the action of drugs relative to such responses that occur in a tissue [23–25]. Scratching a cell monolayer causes specific cell reactions (such as morphology alteration and activation of proliferation and migration) along the wound edge, which may be different from those observed in noninjured or low cell density cultures [23, 43, 44]. The ring assay avoids

some of the problems inherent in the wound model with increased reproducibility and ease of quantification. Single-cell locomotion assays enable cell migration to be distinguished from cell growth. This allows a better determination of the specific effects of drugs on cell migration than a cell population assay, in which drug effects on cell growth may mask or interfere with cell migration. By tracking single cells, the relative proportion of responding cells can easily be evaluated [45]. For the sake of convenience, the vast majority of cell locomotion experiments are performed on 2-dimensional substrates but fail to duplicate in vivo cell behavior [46, 47]. Invasion assays are becoming more popular and include migration through collagen or fibrin or matrix gels. The transwell cell culture chamber is one of the most commonly used tests for chemoattraction and invasion and may be specifically designed for the study of chemotaxis in drug screening. Some studies of the processes of metastasis have also used filters overlaid with a monolayer of endothelial cells or organotypical cultures of tissue sections to test the ability of cancer cells to penetrate into the established cell monolayer or to better mimic in vivo conditions [48–50].

Tumor cell metastasis is a complex and adaptive process highly influenced by the cell environment, and the effects of anticancer drugs must be confirmed in reliable in vivo models before entering the clinical trial phase.

Animal Models of Metastatic Uveal Melanoma

Metastasis is dependent on both the host responses and the intrinsic properties of the tumor cells. During metastatic dissemination, a cancer cell from a primary tumor executes a range of steps, as mentioned above [51, 52]. Animal metastasis models should be designed for mimicking and studying the process of metastasis, although their relevance to the biology of metastasis must be interpreted with caution [53]. There are many ways to establish uveal melanoma liver metastasis models. For example, tumor cell injection using different routes, including intracardiac or intravascular, intrasplenic, direct liver, and intraocular injection, can establish hepatic metastasis models. The selection of a model is determined according to experimental designs.

Posterior Intraocular Melanoma Metastasis Model

Patients with melanoma of the ciliary body or choroid have a poor prognosis and a high incidence of liver metastasis. The human primary uveal melanoma cell lines Mel290, Mel270, OCM8, or 92.1 cells can be xenografted into the ciliary body and choroid to model hepatic metastasis in a mouse or rabbit eye. However, due to the xenograft, either heavy immunosuppressives or immunodeficient animals need to be used. Currently, a murine model with implantation of mouse melanoma B16LS9, Queens, or B16F10 cells in the posterior space (posterior to the lens in the ciliary body or choroid) is able to produce metastases and mimics the process of metastasis from an intraocular tumor. This model may be applied to study the effect of novel antimetastasis agents and the mechanism of metastasis or to discover the sensitivity of early diagnostic indicators. The laboratory of Grossniklaus and colleagues [54] has established the transscleral technique to inoculate melanoma cells into the posterior chamber in a mouse model. This includes the preparation of a tunnel from the cornea at the limbus within the sclera to the ciliary body and choroid with a 30.5-gauge needle under the guidance of a surgical microscope as well as the introduction of tumor cells into the ciliary body and choroid through the needle track via the tip of a 10-μl glass syringe with a blunt metal needle. Both intra- and extraocular tumor growth is achieved in virtually all of the eyes inoculated via the transconjunctival route, and pulmonary metastases develop in a short period of time (7 days). When mouse melanoma B16LS9 cells are injected using this trans-

corneal inoculation technique, the melanoma is confined to the intraocular posterior compartment (PC), and larger intraocular tumors and higher metastasis rates are obtained when compared with the transconjunctival technique [54]. In order to increase the hepatic metastasis rate in murine models, Grossniklaus et al. [55] designed the PC inoculation method in 1995. PC tumors are more similar to human choroidal melanomas. Despite technical difficulties including inadequate visualization and perpendicular inoculation to inject cells, a 50% inoculation rate was achieved. After the injection of tumor cells into the PC of C57BL/6 mice, the pulmonary and presumed liver metastasis rates were 90 and 80%, respectively, 4 weeks after enucleation. PC inoculation generated significantly more hepatic tumor colonies than tail vein injections.

Mouse cutaneous melanoma cell lines have been used in the C57BL/6 host for the study of hepatic metastasis derived from intraocular tumors [53, 54]. Human uveal melanoma cell lines have been inoculated into the eyes of immunodeficient hosts such as athymic nude mice [56] and immunosuppressed rabbits [57]. Using the transscleral technique, the posterior intraocular melanoma model with the human uveal melanoma cell line Mel290 transduced by lentiviral enhanced green fluorescent protein (EGFP) leads to EGFP-expressing micrometastases in the liver 4–6 weeks after inoculation [56]. Specific genetically deficient mice such as pigment epithelium-derived factor knockout mice, interferon gamma knockout mice, and TNF-alpha-deficient mice can be used to form larger hepatic metastases [58]. When immunodeficiency is induced in rabbits by cyclosporin A, 41% of the rabbits developed macroscopical lung metastases and 18% hepatic micrometastases 10 weeks after intraocular inoculation of the primary uveal melanoma cell line 92.1 [57].

Anterior Intraocular Melanoma Metastasis Model

Iris melanomas are less likely to metastasize than ciliary body or choroidal melanomas. In a murine model of mouse melanoma using Queen and B16F10 cells in the anterior chamber (AC), the metastatic rate (33%) was significantly lower than when tumor cells were placed in the posterior chamber (89%). All AC tumors that metastasized to the lungs had also metastasized to the ipsilateral cervical lymph nodes, while no posterior chamber tumors metastasized to ipsilateral cervical lymph nodes [59]. Niederkorn and colleagues [60–63] implanted several different human cell lines (OCM1, OCM3, OCM8, 92.1, Mel202, OMM1, EOM3, and OM431) intracamerally into BALB/c (H-2d) athymic mice. After 50 days, OCM1 and 92.1 induced the highest incidence (80%) of hepatic metastases. Apte et al. [64] inoculated the OCM3 cell line in both AC and PC and found that enucleation had an effect on the number of metastases: the enucleated group gave rise to more hepatic metastases than the nonenucleated group.

Induction of Metastasis by Intrahepatic Injection of Uveal Melanoma Cells

Directly implanting human uveal melanoma cells into the livers of severe combined immunodeficiency (SCID) mice makes it possible to generate different size nodules [65]. When 2×10^7 Mel270 or Mel290 cells or 1×10^7 mouse hepatic metastatic melanoma B16LS9 cells were injected into the liver parenchyma under the capsula in C57BL/6 or SCID mice, hepatic melanoma nodules formed and vasculogenic mimicry patterns were found after 2 and 1 weeks, respectively. This model focuses on critical interactions between the tumor cell and the liver and has been used to screen for serum biomarkers of hepatic metastasis from uveal melanoma [66]; it provides large-size tumors that can be used to estimate the sensitivity of imaging techniques or the response to drugs. Human primary uveal melanoma OCM1a or metastatic uveal melanoma M619 and MUM2B cells are able to form microscopic or expansile hepatic nodules within 2 weeks after the injection of spheroids of tumor cells and metastasize from the liver to the lung within 4–6 weeks [65].

Induction of Metastasis by Intracardiac or Intravascular Injection of Uveal Melanoma Cells

To mimic hematogenous micrometastatic spread, intracardiac or intravascular injection of tumor cells can be applied. Cultured human primary uveal melanoma OCM1 cells with luciferase were injected into the left heart ventricle of BALBc nu/nu mice. The development of metastases was monitored by a bioluminescent reporter imaging system. The results demonstrated that 57% of the mice developed bone metastases in the maxillofacial region [67]. When GFP-expressing Mel290 cells were injected into the tail vein of mice, tiny GFP-positive metastases were found in the liver 28 days after the injection of tumor cells [56].

A substrain of hamster Greene melanoma adapted to grow in the choroid of the rabbit eye has shown to have the tendency to metastasize. Twenty-one normal rabbits were studied for the effects of vascular inoculation of a tumor cell suspension prepared from this melanoma, and 2 untreated rabbits were used for long-term observation. In both groups, metastases to the liver, lung, kidney, and other organs were demonstrated [68].

Liver Metastasis Model Using Intrasplenic Injection

Intrasplenic injection of tumor cells has long been known as an effective method of developing liver metastases in mice and is a successful route to obtain liver metastasis of colon cancer [69, 70]. Human tumor cells have been implanted into the spleen of nude mice. From this injection site, tumor cells gain access to the blood stream and then reach the liver to proliferate into secondary tumor colonies. Hepatic metastases occur with high frequency from a variety of human cancers including uveal melanoma. After 1×10^6 human uveal melanoma OCM8 cells in 100 μ l PBS were transplanted under the spleen capsule in nude mice, tumors with vascularization were generated in the liver within 5–6 weeks. This model is highly reproducible, carries a low mortality, and uses a simple procedure: a 1-cm incision that extends through the skin and abdominal musculature is made at the left subcostal region, and the lower pole of the spleen is exposed. The spleen is then gently exteriorized out of the abdominal cavity for a distance of 1–1.5 cm. Then, 0.1–1 ml tumor cell suspension in PBS is injected through a 27-gauge needle positioned in the spleen through its upper pole. A period of 1 min is allowed to permit all tumor cells to be flushed into the portal circulation, after which time the spleen is removed. Hepatic tumors formed within 4–6 weeks after injection [71]. This method facilitates tumor cells to colonize the liver through the portal circulation system, is applicable for many tumors, and readily lends itself to the evaluation of immunotherapeutic and chemotherapeutic approaches for the treatment of liver metastases; it has also been used to study the mechanisms that lead to hepatic metastases from primary uveal melanoma [67, 72, 73].

Ectopic Metastasis Model

Cells derived from human primary tumors are injected into the subcutis of immunodeficient mice, which results in the formation of a local tumor. Often, the neck is used. In this setting, it was uncommon to observe spontaneous metastasis to distant sites. To generate an ectopic metastasis model, cells derived from hepatic metastases of human uveal melanomas instead of primary uveal melanomas are injected into the subcutis. The advantage of this model is that it is easy to observe and quantify tumor growth. The disadvantages are that it does not represent the process of metastasis, results in different changes of plasma proteinase activity, and deals with an abnormal immune system [74].

Summary of Animal Models of Metastatic Uveal Melanoma

The ideal animal model of hepatic uveal melanoma metastases should faithfully recapitulate the fundamental steps in hepatic metastases from uveal melanomas and should provide

the possibility of examining these steps in vivo. The posterior intraocular melanoma metastasis model is able to form a significant number of metastases, includes all of the steps involved in the metastatic process, and allows the detection and counting of metastatic cells or nodules if the injected uveal melanoma cells have a marker or dye or a reporter gene or are using histology methods. Uveal melanoma cell injection with the use of different routes, including intravenous, intrasplenic, and intrahepatic injection, can establish hepatic metastasis models. In ectopic metastasis models, the outcomes of metastasis might be quite different, since a tumor behaves differently in different tissues, including differences in invasiveness, drug sensitivity, and angiogenesis [67]. However, such models can be suitable for experimental therapeutic purposes, although their relevance to the biology of metastasis must be interpreted with caution [53].

Conclusion

Animal models have served as important vehicles to explore a variety of phenotypes associated with metastatic progression and represent the entire process of metastasis from the growth of the local tumor to the formation of clinically relevant metastasis and the changing microenvironmental cues and interactions that a disseminated cell experiences; nevertheless, they have some limitations, such as ethical concerns regarding animal use, housing costs, and a lack of appropriate models that mimic the precise metastatic stages. Thus, several in vitro cell-based assays that simulate various stages of metastasis, conditions of cancer progression, and microenvironments have been developed and are available to investigate distinct steps of the metastatic cascade and allow for high-throughput drug screening. The in vitro assays are often used in combination with in vivo animal models. Animal models of metastasis have supported drug development, spatiotemporal analysis of the metastatic process by imaging, and use of image data to stage disease, guide tissue sampling from gene array technology, identify metastasis suppressor and promoter genes as novel targets for the development of novel therapies, and help us to understand the complexities of the disease process.

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