A Novel Model of Metastatic Conjunctival Melanoma in Immune-Competent Mice

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PURPOSE. Conjunctival melanoma (CM) is an ocular surface tumor that can lead to fatal metastases. Patients developing, tumor-associated lymphangiogenesis have a significantly increased risk of metastatic disease, because tumor spread primarily occurs via lymphatic vessels to the draining lymph node. Here, we describe a novel immune-competent mouse model of CM that displays tumor-associated lymphangiogenesis with development of metastatic tumors.

METHODS. C57BL/6N mice received C57BL/6N-derived dermal melanoma cells (hepatocyte growth factor [HGF] cyclin dependent kinase-4 [Cdk4]−+) or B16F10 via subconjunctival injection. A clinical score quantified primary tumor growth and metastases were identified by macroscopic examination of the draining lymph nodes, lung, and spleen. Confirmation of tumors and metastases was achieved by immunohistochemical staining for markers of pigmented cells (tyrosinase related protein-2 [TRP2]) and S-100, and of cell proliferation (Ki67). The intra- and peritumoral CD31+ blood and lymphatic vessel endothelium hyaluron receptor-1 (LYVE-1)+ lymphatic vessels were quantified immunohistochemically.

RESULTS. All mice rapidly developed aggressive TRP2+, S100+, and Ki67 + CM. Metastatic tumors were found in the lymph node (9%) and lung (6%) of HGF-Cdk4R24C−treated mice and in the spleen (8%) and lung (17%) of B16F10-treated mice. The amount of peri- and intratumoral blood vessels was significantly increased compared with lymphatic vessels.

CONCLUSIONS. This CM model in immune-competent animals offers new possibilities to study the pathobiology of tumor growth, invasion, and mechanisms of metastatic tumor spread, and provides a robust model to explore new immune-based and antilymphangiogenic treatment modalities of this malignancy.

Keywords: conjunctival melanoma, mouse model, (lymph)-angiogenesis, ocular melanoma, B16F10, hepatocyte growth factor, metastasis

 Conjunctival melanoma (CM) is the second most common malignancy of the ocular surface. The current treatment of primary CM is complete surgical removal of the tumor combined with adjuvant therapy in terms of radio-, cryo-, topical chemo-, and/or immunotherapy. However, even though surgical specimens indicate intact tumor-negative margins and apparent tumor removal, CM patients still display an unusually high rate of local recurrence (36%–62% of patients1−5). Tumor recurrence coincides with a high risk for the development of metastases, which occurs in 12% to 25% of patients.1−3 Metastatic tumors in CM patients are typically first detected in the lymph nodes draining the tumor-containing eye, indicating the primary route of tumor cell dissemination occurs via the lymphatic vessels. In support of this, we previously demonstrated that lymphangiogenesis is associated with the transition of premalignant precursors into CM.7 Moreover, the extent of lymphatic vessels within CM coincided with an increased risk of local recurrence, lymphatic spread of tumor cells, and development of metastatic disease.8−10

Melanomas are derived from the malignant transformation of melanocytes, which are found in a variety of different tissues, including the skin and, within the eye, the uveal tract and conjunctiva. Although all these types of melanoma share a common cellular origin, they can display significantly different disease progression pathways. For example, uveal melanoma spreads from the choroid of the eye via the blood vasculature and metastatic tumors develop almost exclusively in the liver. By contrast, skin and CM spread primarily via lymphatic vessels, and metastatic tumors first develop in local tumor-draining lymph nodes. This suggests that CM is more similar to skin than to uveal melanoma, a pattern that is further supported by data on the genetic mutations associated with these tumors. Uveal melanoma exhibits mutations in GNAQ and GNA11, whereas skin melanoma and CM typically possess BRAF and NRAS mutations.11,12 In addition, the incidence of CM is increasing,1,13−15 which is similar to the increasing frequency observed in skin melanoma.

Primary cutaneous melanomas develop spontaneously in the skin of hepatocyte growth factor (HGF)-cyclin dependent kinase 4...
The basis for this established mouse model of skin melanoma is that the overexpression of a Cdk4R24C mutant impairs the cell cycle control, which furthermore leads to insensitivity of inhibitory signals by INK4A proteins, combined with overexpression of HGF. Hepatocyte growth factor allows maintaining melanocytes within the interfollicular epidermis, next to hair follicles. Adult mice usually do not harbor melanocytes in the interfollicular epidermis, thus this model recapitulates the human situation. Combined HGF-Cdk4R24C expression leads to spontaneous melanoma development and can be accelerated by 7,12-dimethylbenzanthracene (DMBA) treatment or UV irradiation. Subsequently, macroscopically darkly pigmented dermal tumors and metastasis to the lymph nodes and lung are detectable.16

In this study, we used the B16F10 or HGF-Cdk4R24C melanoma cells to develop a novel mouse model of CM that displays tumor-associated lymphangiogenesis with development of metastatic tumors in immune-competent mice, thus allowing exploration of novel immune-mediated therapies.

**MATERIALS AND METHODS**

**Animals and Subconjunctival Injections**

Four groups, each containing 11 to 12 female C57BL/6N mice (8–12 weeks; Charles River Laboratories, Sulzfeld, Germany) received subconjunctival injections of melanoma cells (group 1: 5 × 10⁵ HGF-Cdk4R24C cells, group 2: 1 × 10⁵ HGF-Cdk4R24C cells, group 3: 5 × 10³ HGF-Cdk4R24C, group 4: 5 × 10³ B16F10 cells). For subconjunctival injection, mice were anesthetized with an intraperitoneal injection of a combination of 8 mg/kg body weight ketamine (Ketanest S; Godecke AG, Berlin, Germany) and 0.1 mL/kg body weight xylazine (Rompun; Bayer, Leverkusen, Germany), and both eyes were topically anesthetized with oxybuprocain (conjucain EDO; Dr. Gerhard Mann, Berlin, Germany). Tumor cells (5 × 10⁵/1 × 10⁵ or 5 × 10³ cells/10 μL PBS) were subconjunctivally injected using a Hamilton needle (Hamilton Company, Reno, NV, USA). Correct location of the subconjunctival injection was achieved by appearance of a temporary conjunctival edema, without tumor cell reflux. Injections were performed only on the temporal bulbar conjunctiva in all animals (Fig. 1A). After
**Table.** Clinical Score to Classify Tumor Growth: Mice Were Examined for Chemosis, Redness, and Lid Distance From Bulb

<table>
<thead>
<tr>
<th>Clinical Signs</th>
<th>Chemosis</th>
<th>Redness</th>
<th>Lid Distance From Bulb</th>
<th>Proptosis</th>
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<tbody>
<tr>
<td>Absent</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0 = No</td>
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<tr>
<td>Mild</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<tr>
<td>Moderate</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
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<tr>
<td>Severe</td>
<td>3</td>
<td>3</td>
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<td>Max. 3</td>
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These signs were ranked into four grades: absent, mild, moderate, and severe, and received 0 to 3 points each. Proptosis defined a termination criterion and was only graded yes or no. Proptosis is defined to be so prominent that lid closure was incomplete. Therefore, this criterion counted 5 points. A maximum of 14 points was possible.

**Cell Culture**

The HGF-Cdk4R24C melanoma cells, isolated from cutaneous melanoma of HGF-Cdk4R24C mice on a C57Bl/6 background, were kindly provided by Thomas Tütting (Department of Dermatology, University of Bonn, Bonn, Germany) and originally described by Landsberg et al. The B16F10 cells were kindly provided by Sandra Iden. Cells were kept in RPMI medium (Gibco, Life Technologies, Darmstadt, Germany), containing 10% fetal calf serum, Pen Strep (both PAA medium (Gibco, Life Technologies, Darmstadt, Germany), C3H-10T1/2 (BD Pharmingen, Heidelberg, Germany), and lymphatic vessel endothelium hyaluronan receptor 1 (LYVE1) (AngioBioCo, Del Mar, CA, USA). The primary antibodies were added in a dilution of 1:100 in PBS (containing 2% fetal calf serum) (overnight incubation, 4°C). The next day, samples were washed three times for 5 minutes with PBS with continuous shaking. Primary antibodies were detected with corresponding secondary antibodies (2 hours at 20°C), diluted in 2% normal goat serum (Dako, Glostrup, Denmark) in PBS to avoid nonspecific binding.

**Statistical Analyses**

Statistical analysis was performed using Prism 6, V6.02 (GraphPad Software, San Diego, CA, USA). A two-tailed unpaired t-test was used for detection of differences between the groups. For the survival curve, a log-rank test was performed. For the analysis of blood and lymphatic vessels, a Kruskal-Wallis test was used. P values less than 0.05 were considered statistically significant.

**Results**

**Subconjunctival Injection of HGF-Cdk4R24C Melanoma Cells Induced Rapid and Aggressive CM Growth in All Animals**

A major aim was to develop a novel robust mouse model for CM. First, we tested different amounts of injected tumor cells and followed the kinetics of tumor formation and progression. We injected $5 \times 10^5$, $1 \times 10^5$, or $5 \times 10^4$ cells of C57BL/6N-derived HGF-Cdk4R24C melanoma cells into female C57BL/6N mice. All animals showed a rapid and aggressive local tumor growth with...
darkly pigmented solid tumors (Figs. 1B–D). At the end of the first week, an intense vascularization was visible (Fig. 1B), and after 7 to 10 days a brown tumor was visible. The mice had to be killed due to tumor growth causing massive unilateral proptosis with lid closure difficulties. For example, when $5 \times 10^5$ cells were injected, the mice had to be euthanized within approximately 9 days. In less pigmented areas, an intense neovascularization was visible (Fig. 1E). The more HGF-Cdk4R24C cells were injected, the faster was the tumor growth. The clinical findings were quantified using a clinical score (Table; Fig. 1F), demonstrating that high numbers of tumor cells led to earlier and higher clinical scores. Figure 1G shows the survival rates of the HGF-Cdk4R24C groups, using proptosis as an endpoint. The survival curve similarly shows that high numbers of tumor cells led to earlier termination of the experiments ($P < 0.0001$): all mice were killed by day 9 in the $5 \times 10^5$ group, whereas in the low-dose group ($5 \times 10^3$ cells) mice could be kept until day 21.

HGF-Cdk4R24C- and B16F10-Induced CM Show a Similar Ocular Phenotype

In a next step, we compared the tumor growth kinetic of the HGF-Cdk4R24C cells with B16F10 cells. In these experiments, we injected 5000 HGF-Cdk4R24C cells as well as 5000 B16F10 cells and compared tumor growth, clinical scores, and survival (defined by incomplete lid closure due to large tumors). Exenterating the tumor-bearing eye revealed tumor infiltration of the whole orbit in B16F10- or HGF-Cdk4R24C–induced CM (Figs. 2A–C). Regarding the clinical scores, the B16F10-induced CM was slightly faster than the HGF-Cdk4R24C–induced CM (Fig. 2D). The survival curves did not significantly differ from each other (Fig. 2E) ($P = 0.075$). These data indicate significant and aggressive growth of both HGF-Cdk4R24C and B16F10 tumor cells in the conjunctiva of the eye with a higher speed of tumor growth for B16F10 compared with the HGF-Cdk4R24C–induced CM.

HGF-Cdk4R24C- and B16F10 Melanoma Cells In Vivo Express Tumor Markers S100, Tyrosinase-Related Protein 2 (TRP2), and Ki67

After development of the tumor, we verified the melanoma properties of the HGF-Cdk4R24C and B16F10 cell line and validated the known melanoma profile (i.e., expression of TRP2, S100, and the proliferation marker Ki67). For this, tumor-containing eyes were harvested, cryosectioned, and stained by immunohistochemistry for these markers. The tumors were contained within the conjunctiva and the globe was microscopically intact. In some cases, the bulbus was compressed by the tumor mass (data not shown). HGF-Cdk4R24C tumor cells expressed high levels of TRP2, Ki67, and S100 (Figs. 3B–D). Similar results were obtained for the B16F10-induced tumors (Figs. 3E–G), whereby S100 was weakly expressed here.

In HGF-Cdk4R24C- and B16F10-Induced CM, the Tumor Is Enriched With CD31+ Blood and LYVE1+ Lymphatic Vessels

Progressive tumor growth coincided with intra- and peritumoral LYVE1+ lymphatic vessels and CD31+ blood vessels (Fig. 4). Quantification of the areas covered by CD31+ blood vessels...
or LYVE1+ lymphatic vessels showed that the intra- and peritumoral amount of blood vessels was significantly increased compared with lymphatic vessels, both in the HGF-Cdk4R24C– (intratumoral \( P < 0.05 \) and peritumoral \( P < 0.01 \), respectively; Figs. 4A–C) and B16F10-induced CM (intratumoral \( P < 0.0001 \) and peritumoral \( P < 0.0001 \), respectively; Figs. 4D–F). Comparing the location within the tumor, lymphatic vessels were higher in the tumor periphery than in the tumor center, both in the HGF-Cdk4R24C– (not significant) and in the B16F10-induced CMs (\( P < 0.01 \)). Blood vessels were detected throughout the tumor (Figs. 4B, 4E). There was a tendency for more lymphatic vessels in the periphery of the HGF-Cdk4R24C– group compared with the B16F10 group. However, these results were not significantly different (\( P > 0.05 \)).

**Lymphatic Metastasis in the Draining Lymph Node and Distant Metastasis to the Lung in HGF-Cdk4R24C–Treated Mice**

In total, 23% of mice treated with \( 5 \times 10^3 \) or \( 1 \times 10^5 \) HGF-Cdk4R24C– cells showed macroscopically detectable darkly pigmented spots in the draining lymph nodes (lymph node: 14% of the lower dosed groups or a total of 9% in all groups; lung: 9% of the lower dosed groups or a total of 6% in all groups) (Fig. 5). Compared with the normal contralateral lymph nodes (Fig. 5A), the tumor-containing eye draining lymph nodes were pigmented and enlarged (Fig. 5B). Mice that had been injected with \( 5 \times 10^5 \) or more cells showed intense pigmentation of the draining lymph nodes, but without a clear demarcation zone (data not shown). None of the animals that received \( 5 \times 10^5 \) cells showed metastasis, likely because the rapid growth of the primary tumor required early termination of the experiment. Decreasing the tumor cell dose from \( 5 \times 10^5 \) cells to \( 5 \times 10^3 \) or \( 1 \times 10^5 \) and thereby prolonging the experiment led to metastases (see Fig. 5B as an example for a large metastasis in the draining lymph node). The presence of metastatic tumor cells was confirmed by expression of S100 (Fig. 5C) and TRP2 (Figs. 5D, 5E). Dark-pigmented spots on the lung were observed macroscopically, and immunohistochemical analysis confirmed positivity for TRP2 and S100 (Figs. 5F–K).

**Lymphatic Metastasis to the Spleen and Distant Metastasis to the Lung in B16F10–Treated Mice**

Mice treated with \( 5 \times 10^3 \) cells of B16F10 mice developed metastasis to the spleen (8.3%) and to the lung (17%). The metastatic tumor was confirmed histologically by positive staining for TRP2 (Figs. 6A–E). The S100 staining was nearly absent. In the spleen, only one large-sized brown pigmentation was detectable (Fig. 6A), whereas in the lung, multiple small pigmented dots (Fig. 6D), all positive for TRP2 (Fig. 6E), were observed. Metastasis to the lymph node was not observed in the B16F10 group.

**DISCUSSION**

We have previously shown the importance of peri- and intratumoral lymphatic vessels for recurrence, metastasis, and prognosis in different ocular malignancies, specifically in CM.\(^8\)\(^-\)\(^10\)\(^,\)\(^22\)\(^-\)\(^26\) Therefore, antilymphangiogenic therapies are needed to develop new therapeutic options. To study them, we need murine immune-competent models because for
lymphangiogenesis, immune cells are closely interlinked. However, to date there are no immune-competent mouse models to study the biology of CM and to test new therapeutic approaches. In this study, we aimed to develop a new model for CM in immune-competent mice. In patients, CM growth is initiated within the epithelium and invasion across the basement membrane is a major indicator of malignancy. In our model, we are directly injecting tumor cells underneath the basement membrane, to mimic the clinicopathological situation. Similar to existing tumor models in other anatomical sites of the eye, this mouse model has its pros and cons. A major disadvantage might be the use of cutaneous and not primary CM cells, as it is not suitable for testing human antibodies or immune-mediated human therapies. However, due to the current lack of mouse CM cell lines and considering the fact that epidermis and conjunctiva show many similarities regarding embryonic origin, development, stratification, lymphatic drainage, and outer barrier to the environment, we consider cutaneous cell lines are currently one of the best options for tumor induction in immune-competent syngeneic mice. A similar approach for uveal melanoma is used in a mouse model developed by Lattier et al. and Dithmar et al., in which B16F10 cutaneous melanoma cells are injected in the posterior compartment of the eye. Another approach has been performed by De Waard et al. Hereby, the authors inject human CM cells into severe combined immunodeficient (SCID) mice.

As noted above, an advantage of our allogeneic model reported here is that the tumor develops in healthy, immune-competent animals, and does not require genetic modifications or inactivation of the immune system, in contrast to the xenograft approaches, in which human tumor cells are orthotypically transplanted in SCID mice or athymic nude mice. It is well accepted that the tumor microenvironment is essential for progression of tumors, therefore our model presented here allows studying the effect of the tumor microenvironment on the host’s immune system, neovascularization, and metastasis.

The role of different murine immune cells, cytokines, or growth factors can be studied in detail in this model (e.g., cancer-associated fibroblasts or macrophages, chemokine receptors, or the origin of tumoral lymphatic vessels). We have optimized the protocol for a mouse strain that is also commonly used to generate genetically modified mouse strains, opening the possibility to apply our model using specific mutant mice in future studies. In humans, genetic causes underlying CM are poorly investigated. Another advantage is that tumor growth is fast and aggressive, allowing quick and cost-reduced experiments. The aggressive tumor growth imitates the clinical situation in patients exemplified by a full infiltration of the orbit, which is comparable to a T3 stage in humans. However, orbital infiltration is clinically seen much less frequently than in our murine model, which underlines a disadvantage of this model: it is not directly comparable to the clinical situation. This is also seen in the metastasis behavior: our model provides faster manifestation of metastasis compared with humans.

The tumor growth and metastases in our model developed within approximately 14 days in the draining lymph nodes or the lung, a faster process than seen in an immune-deficient conjunctival mouse model. In our model, tumor growth is much faster, although only 0.005% of the number of cells were injected compared with the immune incompetent model. This emphasizes the importance of a functional immune system that is used by the tumor to promote and support the malignancy.

**Figure 4.** Quantification of peri- and intratumoral blood and lymphatic vessels: (A) In HGF-Cdk4 R24C-induced CM, more peri- and intratumoral blood vessels (BVs) than lymphatic vessels (LVs) are detectable (P < 0.05 and P < 0.01). No significant differences were seen between peri- and intratumoral lymphatic vessels. (B) Example of intratumoral BVs (white arrowheads) and LVs (red arrows). (C) Example of a large peritumoral LV. (D) In B16F10-induced CM peri- and intratumoral clearly more BVs than LVs were measured (P < 0.0001). Intratumorally, very few lymphatic vessels were detected compared with peritumoral (P < 0.01). (E) Example of intratumoral BVs and LVs. (F) Example of peritumoral BVs and LVs, showing LVs mainly located at the tumor margin.
FIGURE 5. HGF-Cdk4R24C CM metastasizes to the draining lymph node and lung: (A) Control lymph nodes with no apparent brown pigmentation. (B) Lymphatic metastasis to the draining lymph nodes associated with slow-growing conjunctival melanoma, induced by injection of 5000 or 100,000 cells. (C) Immunohistochemical analysis of cryosectioned lymph node metastases revealed positivity for S100 and (D, E) TRP2. Scale bar: 50 μm. (F, G) Macroscopic appearance of distant lung metastasis. (H–K) Immunohistochemistry confirmed the expression of S100 and TRP2 in the lung metastasis of CM. These data are representative for two animals with a total of 21 lung metastases.

FIGURE 6. B16F10 CM metastasizes to the spleen and lung: (A) Spleen with a brown pigmentation (arrow). (B) Hematoxylin and eosin staining of the pigmented area in the spleen. (C) TRP2 positivity of the spleen cells. (D) Lung metastasis seen as multiple small brown spots. (E) Pigmented cells are positive for TRP2.
Still, both models are usable for different questions and complement one another. The reason for this quick metastatic spread is unclear. Clinically, metastases are often detected months or years after the resection of the primary malignancy.4,14,15 In human studies, regional lymph node metastasis appeared within 34 ± 10 months after primary diagnoses of CM.8 Distant metastases were observed with a mean time interval of 42 ± 10 months after primary treatment.8

In the current study, we used two skin melanoma tumor cell lines to develop a new mouse model of CM, which is critical for the development and testing of new therapies as well as studying the mechanisms of disease progression. A key requirement of a useful CM model is that the growth of primary tumors coincides with lymphangiogenesis, lymphatic tumor spread, and development of local draining lymph node metastases, to mimic human disease.

In conclusion, we established a mouse model of CM in immune-competent C57/BL6N mice, with reproducible primary tumor growth as well as locoregional lymphatic and distant metastasis. This allows further studies of the biology, microenvironment, hem- and lymphangiogenesis, and immunity in this important form of ocular melanoma. Furthermore, this immune-competent in vivo model provides valuable tools to identify effective antitumor therapies both locally in the conjunctiva as well as at locoregional and distant metastatic organ sites based on immunomodulatory and antilymphangiogenic strategies. However, all future conclusions resulting from this mouse model will still need verification in the clinical situation to improve the outcome for patients who suffer from CM.

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References


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