Expression of Multidrug Resistance Transporter ABCB5 in a Murine Model of Human Conjunctival Melanoma

Nadine E. de Waard, Paraskevi E. Kolovou, Sean P. McGuire, Jinfeng Cao, Natasha Y. Frank, Markus H. Frank, Martine J. Jager, Bruce R. Ksander

Department of Ophthalmology, Schepens Eye Research Institute and Massachusetts Eye and Ear Infirmary, Division of Genetics, Brigham and Women’s Hospital, and Harvard Stem Cell Institute, Harvard Medical School, and Transplant Research Program, Division of Nephrology, Boston Children’s Hospital, Boston, Mass., USA; Department of Ophthalmology, LUMC, Leiden, The Netherlands; Department of Ophthalmology, The Second Hospital of Jilin University, Changchun, China

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Conjunctival melanoma · ABCB5 · Stem cells

Abstract
Conjunctival melanoma (CM) is a rare ocular malignancy with a high tendency to reoccur locally and with a high risk of metastatic disease. Metastases are often unresponsive to conventional treatment. Recently, an animal model was set up using human CM cells. Orthotopic xenografts from human CM were created by subconjunctival injection of three different CM cell lines into NOD.Cg-Prkdc<sup>scid</sup> IL2rg<sup>tm1Wjl</sup>/SzJ (NSG) mice. Subconjunctival injection of cultured CM cells led to excellent subconjunctival growth, but no metastases were found. When single-cell suspensions were obtained from the subconjunctival xenografts and passaged in vivo, all mice developed metastases. As recent findings indicate that cancer stem cells are linked to tumor recurrences, we used this new murine model to determine the expression of the stem cell marker ABCB5 during tumor progression. Expression of the ABCB5 protein was determined in three cell lines and during different stages of tumor development as observed in our model. All three cell lines contained a subpopulation of cells positive for ABCB5. During tumor development, expression of ABCB5 increased during phases of tumor expansion. Furthermore, expression of ABCB5 was increased in metastases. Using this model for CM, we were able to initiate metastatic spread and determine the expression of the stem cell marker ABCB5 during different stages of tumor development, identifying ABCB5 as a potential novel therapeutic target. This study illustrates the potential of our newly established murine model.
Introduction

 Conjunctival melanoma (CM) is an extremely rare ocular malignancy that originates from melanocytes residing in the conjunctiva. Current therapies focus on local excision with additional therapeutic modalities, such as cryotherapy, plaque therapy, and topical chemotherapy, being used to target residual tumor cells [1–4]. The success of treatment varies among hospitals; however, a common problem encountered is the tendency of CM to recur locally [5]. Recurrences are associated with an increased risk of metastatic disease often unresponsive to conventional treatment. The mortality rate following the diagnosis of CM is 15% after 5 years and 30% after 10 years, with metastatic disease as the main cause of death [6]. Therefore, more effective treatments are needed in order to prevent local recurrence or the development of metastases. A murine model that follows the natural progression from primary tumor to metastatic disease will enable the investigation of novel therapeutic approaches. We have very recently developed a human-to-mouse xenogeneic tumor model for CM by using immunodeficient NOD.Cg-Prkdc<sup>scid</sup>IL2rg<sup>tm1Wjl</sup>/SzJ (NSG) mice [7].

 In several malignancies, a subpopulation of cells known as cancer stem cells (CSC) is linked to recurrence and disease progression [8, 9]. The presence of CSC limits the therapeutic effect of treatments, since they appear to be refractory to treatment. These CSC have the potential of extensive proliferation, differentiation and maintenance of tumor growth, despite the fact that they comprise only a small fraction of neoplastic cells [8, 10–12]. As a result, failure of targeted elimination of these so-called quiescent CSC leads to recurrence of the disease [13]. Of particular interest is the stem cell marker ABCB5, an ATP-binding cassette (ABC) multidrug resistance transporter, which also mediates cell fusion, stem cell function, and vasculogenic plasticity [14]. Cutaneous melanoma is enriched for a subpopulation of cells positive for ABCB5, and ABCB5 has been identified as a molecular marker for melanoma progression, with a distinct subpopulation of ABCB5+ cells displaying increased tumorigenicity [8, 10–13, 15]. Moreover, ABCB5+ cells have been shown to play an active role in conferring chemoresistance through the efflux function of ABCB5 [16, 17]. It becomes therefore highly relevant to determine whether CM also possesses a subpopulation enriched for ABCB5, and if the presence of ABCB5 identifies a population of tumor cells with increased tumorigenicity, since this will have fundamental implications for therapy. If ABCB5+ CSC are indeed present in CM, new treatments may be identified based on the ability to target these cells [11, 18]. Since we have recently established a new murine model for human CM [7], we examined the expression of ABCB5 in three different CM cell lines to identify the role of ABCB5 in CM development and disease progression.

Cell Culture

 Three CM cell lines were used, and all were derived from locally recurrent tumors. Cell lines CRMM-1 and CRMM-2 were created by Dr. G. Nareyek (Essen, Germany) [19] and kindly provided by Dr. M. Madigan (Sydney, N.S.W., Australia). The cell line CM2005.1 has been created by Dr. S. Keijser (LUMC, Leiden, The Netherlands) [20]. Cells were grown under standard conditions [7]. As many new drug studies target specific mutations, we determined the presence of conjunctival melanoma-specific mutations in these cell lines. DNA material from the three CM cell lines as well as paraffin-embedded tumors of CM xenografts were extracted with the QIAamp DNA mini kit (Qiagen, Venlo, The Netherlands) and the ReliaPrep FFPE gDNA miniprep System (Promega, Fitchburg, Wis., USA), respectively. PCR analysis was performed in order to amplify BRAF exon 15, NRAS exon 2, and NRAS exon 3. Following PCR, the reverse primer was added to the purified PCR products. Sequencing was examined (Base-
clear, Leiden, The Netherlands), and mutations were identified using SnapGene software (GSL Biotech LLC, Chicago, Ill., USA) [7]. The BRAF c.1799T>A (V600E) mutation was detected in both of the cell lines CRMM-1 and CM2005.1, as well as their established xenografts and in vivo passaged tumors. The cell line CRMM-2 and the corresponding xenografts harbor an NRAS c.182A>T (Q61L) mutation [7].

Establishment of CM Xenografts

NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/SzJ (NSG) – nonobese diabetic/severe combined immunodeficiency (NOD/SCID) IL-2 receptor gamma chain null mice – were obtained from the Jackson Laboratory, Bar Harbor, Maine, USA. All mice were maintained under defined conditions as stated in the institutional guidelines of the Schepens Eye Research Institute. Experiments were performed in accordance with experimental protocols following the guidelines for the use of animals in research of the Association for Research in Vision and Ophthalmology. Anesthesia was accomplished by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (9 mg/kg). Mice were sacrificed using CO₂ asphyxia followed by cervical dislocation. At the time of autopsy, all internal organs were inspected macroscopically, paying special attention to the regional lymph nodes, heart, lung, and liver.

Orthotopic xenografts of human CM were developed by subconjunctival injection of the cell lines CRMM-1, CRMM-2, and CM2005.1. The injected cells were harvested from in vitro cultures; cells were counted, washed, and resuspended in culture media. Mice were anesthetized as described above, and injections were performed by microscopy using a 33-gauge needle inserted into the nasal subconjunctival space. Each injection contained a total of 0.4 × 10<sup>6</sup> cells in a volume of 5 μl. Tumor development and growth was monitored weekly, at least up to the end point of 10 weeks, unless excessive tumor growth required early sacrifice. In vivo passaging of the primary tumor xenografts was performed by surgical dissection of the tumor xenografts, after which a single-cell suspension was prepared. Cells were counted, rinsed, and centrifuged, which was followed by resuspension in culture media. Subsequently, orthotopic xenografts of in vivo cultured CM cells were established as described above (fig. 1) [7]. Expression of ABCB5 on xenografts and metastases was determined by fluorescence-activated cell sorting on isolated cells.

Expression of the ABCB5 Protein in Human CM Cell Lines

To determine the presence of ABCB5+ cells, we used the IgG1κ mAb 3C2–1D12 [21], with MOPC-31C mouse isotype control mAb (Sigma-Aldrich, St. Louis, Mo., USA) as control. As secondary antibodies, we used Alexa Fluor (AF) 488-conjugated goat anti-mouse IgG, AF 594-conjugated goat anti-mouse IgG, and AF 694-conjugated goat anti-mouse IgG (Invitrogen). To confirm staining specificity, we used a rabbit anti-ABCB5 pAb (Novus, Littleton, Colo., USA). Surface ABCB5 expression on cultured cells was determined by single-color flow cytometry with anti-ABCB5 mAb. Cells were washed with Hank’s Balanced Salt Solution (HBSS, Lonza) before detachment with Cell Stripper (Cellgro, Herndon, Va., USA). The cells (1 × 10<sup>6</sup> cells/sample) were incubated for 30 min at 4 °C with 2 μg/ml of anti-ABCB5 mAb followed by counterstaining with a secondary goat anti-mouse IgG antibody. Washing with 2% FBS/PBS (FACS) buffer was done after each step.

Expression of ABCB5 was determined by flow cytometry in three cell lines that had been derived from three human CM (fig. 1). We observed ABCB5+ cells in all three cell lines, with up to 0.1% of cells staining for ABCB5 (CRMM-1: 0.04–0.09%, CRMM-2: 0.03–0.1%, CM2005.1:
The presence of the ABCB5+ subpopulation was also confirmed using another commercially available antibody, a rabbit anti-ABCB5 pAb (Novus), which stained the same number of cells (0.05% of total, data not shown). These results indicate that all three cultured CM cell lines contain a small but clearly defined population of ABCB5+ cells.

**ABCB5 Expression during Orthotopic Tumor Development**

Cultured cells were injected subconjunctivally into NSG mice. All three cell lines, CRMM-1, CRMM-2, and CM2005.1, induced tumor growth in 33/33, 34/34, and 35/35 mice, respectively, showing excellent growth [7]. We examined the expression of ABCB5 in several subconjunctival tumors that were harvested at 3, 6, and 9 weeks after inoculation using FACS analysis. All primary xenografts contained a subpopulation of ABCB5+ cells (fig. 2c), with a statistically significant difference in ABCB5 expression between cell lines CRMM-1 and CRMM-2 and their respective tumors at 3 and 6 weeks (p < 0.05). Moreover, a statistically significant difference in ABCB5 expression was also observed between the tumors isolated at 3 or 6 weeks and those isolated at 9 weeks (p < 0.05). However, none of the mice developed any metastases at any time point.

In order to select for more aggressive tumor cells, we used in vivo passaging. A single-cell suspension was generated from CM xenografts and subsequently transplanted into the subconjunctival space of another set of immunodeficient NSG mice. We chose to use tumors developed from the cell lines CRMM-1 and CM2005.1 for this set of experiments, as both showed a similar in vivo growth rate. All mice that had been injected with tumor cells developed a visible subconjunctival tumor within 2 weeks (CRMM-1: n = 9, CM2005.1: n = 12). The percentage of ABCB5+ cells on single-cell suspensions generated from the tumors used for in vivo passaging was less than 0.06%.
ABCBS5 Expression in Distant Metastases

About 7–8 weeks after in vivo transfer of tumor cells, the mice lost weight and started showing symptoms of pain. We therefore decided to sacrifice the mice 9 weeks after injection. Autopsy showed that all of the mice that had received a transfer of subconjunctival cells developed metastases (CRMM-1: n = 9, CM2005.1: n = 12). The metastases were found mainly...
in the cervical lymph nodes, heart, and lungs [7]. We examined the presence of ABCB5+ cells in the metastases by creating single-cell suspensions from the various metastases found at the time of autopsy, and the metastases were dissected under a microscope. As the metastases were pigmented tumors, we were able to separate them from their environment. We then used a mechanical tissue divider to create a cell suspension. The cell suspension was subsequently filtered, washed, counted, and used in FACS analysis. We used the IgG1κ anti-ABCB5 mAb (clone 3C2–1D12). Metastases in the heart (n = 3) and cervical lymph nodes (n = 5) showed an ABCB5 expression of 0.63 and 0.28%, respectively, while ABCB5 expression in lung metastases was even higher at ∼2% (fig. 2d, e). We can therefore conclude that the expression of ABCB5 in the metastases is increased compared to the primary tumor.

Discussion

It has long been known that CM has a high tendency to reoccur locally and may lead to lymphatic and distant metastases. However, the lack of a reliable animal model has hampered the true understanding of the underlying mechanisms [22, 23]. Furthermore, over the past few years, studies in different types of cancer have identified phenotypically distinct subpopulations of tumor cells based on the expression of different markers, among them ABCB5, an ABC transporter. ABC transporters are engaged in a wide variety of physiological processes [24] and have been associated with multidrug resistance [25]. ABCB5 expression in particular has been demonstrated to confer chemoresistance through the efflux function of the ABCB5 transporter and to correlate with clinical tumor progression of invasive melanoma in a competitive tumor development model [12]. Additionally, recent studies have shown that the presence of these stem cell markers may be responsible for enhanced tumorigenicity, local recurrences, and the development of clinically significant metastasis [26–29].

We based our hypothesis that CM harbors a subpopulation of ABCB5+ cells on previous work on cutaneous melanoma, which showed that cutaneous melanoma contains an ABCB5 subpopulation exhibiting stem cell-like properties [12, 30]. Due to the assumed similarities between cutaneous melanoma and CM and the availability of the specific IgG1κ anti-ABCB5 mAb, we decided to explore the possible expression of ABCB5 in CM and used our recently established human CM xenograft model to observe these cells in vivo in primary as well as metastatic disease. Our data demonstrate that all three cell lines, originated from CM [19, 20],
possess a subpopulation of cells positive for the marker ABCB5. In all analyses, we observed a small but clearly defined population of ABCB5+ cells [31, 32].

To further investigate whether or not the expression of ABCB5 in the CM cells would be influenced by the in vivo environment, we analyzed tumor xenografts using the same cell lines through flow cytometry. We observed an increase in the expression of ABCB5 during the early stages of tumor development in primary tumors. However, when the tumors had grown, the expression of ABCB5+ cells subsequently decreased to the original levels found in the cell lines at the time of injection, suggesting that the stem cell marker ABCB5 plays an important role in early tumor formation and establishment. It is of interest though that in the metastatic tumors, the levels of ABCB5 expression were higher than those of the cell lines or original xenografts, indicating that ABCB5 may play a role in metastatic spread.

While we demonstrated that the severely immunodeficient NSG mice allowed successful engraftment of all subconjunctivally placed xenografts and the formation of metastasis, it remains to be determined whether or not the ABCB5+ cells are indeed responsible for the high rates of local recurrences in human CM. Further studies are needed to clarify this. Further, whilst we have shown that the use of NSG mice represents a major advance in the establishment of a mouse model for CM due to the high susceptibility of these mice to human tumor cell growth, the lack of a functional immune system in mice limits the possibilities for effective studies to investigate the concept of CSC in general and ABCB5+ stem cells in particular in this model.

In conclusion, we described the establishment of a murine model for CM, which we used to analyze the expression of the specific marker ABCB5 during different stages of tumor development. We identified the presence of ABCB5, a novel marker for a small, potentially therapy-resistant, subpopulation of CSC in CM. Furthermore, using our established murine model for CM, we obtained metastases after in vivo passaging and showed that ABCB5 is expressed during early tumor development, which increases even more in distant metastases, suggesting a definite role in metastatic potential. It remains to be seen whether ABCB5 confers treatment resistance in CM, and this is the subject of other studies, which are already underway. Given the fact though that ABCB5 isoforms have been shown to confer chemotherapy resistance to multiple treatment agents in melanoma and hepatocellular carcinoma [33–36], this further highlights the importance of our animal model and its use for testing different therapeutic modalities that would target the reversal of ABCB5 resistance and lead to the eradication of these ABCB5+ CM cells.

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References


Erratum

In the article by de Waard NE, Kolovou PE, McGuire SP, Cao J, Frank NY, Frank MH, Jager MJ and Ksander BR, entitled ‘Expression of Multidrug Resistance Transporter ABCB5 in a Murine Model of Human Conjunctival Melanoma’ [Ocul Oncol Pathol 2015;1:182–189, DOI: 10.1159/000371555], the following statement needs to be added:

Nadine E. de Waard and Paraskevi E. Kolovou contributed equally to this paper and are both considered first authors.