Malignant melanomas of the ocular surface (conjunctival melanoma [CM]) and within the eye (uveal melanoma [UM]) show different metastatic behavior. Conjunctival melanoma has a propensity to first spread into regional lymph nodes. On the other hand, UM spreads almost exclusively via hematogenic route to the liver. We investigated whether these different metastatic patterns might be attributable to differential hem- and lymphangiogenic characteristics of CM and UM cells.

METHODS. Human CM (CM2005.1, CRMM1, CRMM2) and UM (Mel270, Mel290, OM431) cell lines were analyzed for VEGF-A, -C, and -D expression by RT-PCR and ELISA. The influence of CM- or UM-conditioned medium on blood (BEC) and lymphatic (LEC) endothelial cell proliferation and migration was measured using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) and scratch assays, respectively.

RESULTS. Vascular endothelial growth factor-A, -C and -D mRNA, and VEGF-A and -D protein were expressed by all CM and UM cell lines, while VEGF-C protein was only expressed by UM cell lines. The CM- and UM-conditioned medium did neither differentially affect BEC (P = 0.86) proliferation, nor BEC (P = 0.56) and LEC (P = 0.90) migration.

CONCLUSIONS. Conjunctival melanoma cell lines did not show a higher prolymphangiogenic potential, and UM cell lines did not show a higher prohemangiogenic potential. Accordingly, other mechanisms within the tumor microenvironment might account for the diverging metastatic patterns of conjunctival versus uveal melanomas.

Keywords: conjunctival melanoma, uveal melanoma, hemangiogenesis, lymphangiogenesis, VEGF
The purpose of the present study was to analyze whether a different hem- and lymphangiogenic capacity of CM and UM cells contributes to the different metastatic behaviors of conjunctival and uveal melanomas. Therefore, we studied VEGF-A, -C, and -D expression in human CM and UM cell lines as well as the impact of these cell lines on blood (BEC) and lymphatic (LEC) endothelial cell proliferation and migration.

**METHODS**

**Cell Lines and Culturing Conditions**

Human CM cell lines (CM2005.1, CRMM1, and CRMM2) and UM cell lines (Mel270, Mel290, and OM431) were used for the experiments. Cell line CM2005.1 was isolated by de Keizer et al. CRMM1 and CRMM2 were isolated by Nareyek et al. Mel270 and Mel290 were isolated by Chen et al. and Verbik et al. All CM cell lines used for the present work were kindly provided by one of the authors (MMJ), and all UM cell lines were kindly provided by Bruce R. Ksander (Scheepens Eye Research Institute, Boston, MA, USA). Cell line CM2005.1 derived from a primary tumor originally located at the palpebral conjunctiva of the upper eyelid, and was isolated from a local recurrence appearing 5 years after initial tumor removal. The tumor displayed a spindle-cell type, and immunohistochemical staining was positive for melanocytic markers S-100, MelanA, and HMB 45. Cell line CRMM1 derived from a tumor originally located in the bulbar conjunctiva and was isolated from a local recurrence. Cell line CRMM1 showed a high expression of tumor marker HMB-45 and displayed spindle-cell morphology. Also, CRMM2 cells derived from a tumor originally located at the bulbar conjunctiva and was isolated from a recurrent tumor showing high HMB-45 expression and displaying a spindle-cell type. All tested CM cell lines were shown to have either BRAF (CRMM1, CM2005.1) or NRAS (CRMM2) mutations.

Uveal melanoma cell line Mel270 derived from a ciliary body melanoma, which received neoadjuvant brachytherapy (I125 plaque) before enucleation. Hepatic metastases occurred 3 years after removal of the primary tumor. Cell line Mel290 was isolated from a primary uveal melanoma. Cell lines Mel270 and Mel290 showed no BRAF, NRAS, KRAS, or HRAS mutations of the genomic DNA. A GNAQ mutation was detected in Mel270, but not in Mel290. Cell line Mel270 is composed primarily of spindle cells, while Mel290 shows epithelioid cell morphology. Cell line OM431 was isolated from a recurrent epithelioid cell type choroidal melanoma. Genetically, OM431 revealed a BRAF mutation and a GNAQ and GNAI1 wild-type.

Tumor cell lines were cultured in melanoma medium, composed of Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies, Darmstadt, Germany) with 1% pen-strep/L-glutamine, 1% sodium pyruvate, 0.4% 2-β-mercaptoethanol (PAA Laboratories, Pasching, Austria) and 10% fetal bovine serum (FBS; Life Technologies). To generate tumor cell-conditioned medium, melanoma medium containing only 1% FBS (Life Technologies) was used. Blood endothelial cells and lymphatic endothelial cells (Promocell, Heidelberg, Germany) were cultured in Endothelial Cell Growth Medium MV2 Kit with all supplied supplements (Promocell), which contained additionally 1% pen-strep/L-glutamine (PAA Laboratories). For proliferation assays, minimal endothelial medium was used, supplemented only with 1% fetal calf serum (FCS; Promocell). Cell lines were cultured with 20% O2, 5% CO2, and 37°C, and were passaged when 80% to 90% confluent. Cell lines were subcultured with Trypsin/EDTA (0.04%, 0.03%) and Trypsin Neutralizing Solution (TNS; 0.05% Trypsin Inhibitor in 0.1% BSA; Promocell).

**Tumor Cell–Conditioned Medium**

Tumor cell lines were seeded at a density of 1 x 10⁵ cells/mL in melanoma medium. After 24 hours the medium was changed to minimal melanoma medium and the cell lines were incubated with 1% O2, 5% CO2 at 37°C in a CO2-Incubator with O2 control (CO2-Incubator CB 150; Binder, Tuttinglen, Germany). Culture supernatant was collected after 24 hours, centrifuged for 10 minutes at 500g, and stored at −20°C until use. Stagnant (incubated under 37°C and hypoxic conditions), but unconditioned melanoma medium without supplements was used as control. Equal parts of tumor cell culture supernatant and minimal endothelial cell medium were mixed to create tumor cell-conditioned medium to be used in LEC/BEC proliferation and migration assays. The collection of supernatants was carried out for each melanoma cell line three times using different cell passages.

**mRNA Expression**

Cell lines were cultivated while producing conditioned medium. The mRNA of melanoma cell lines was isolated after 24 hours of culture in minimal melanoma medium and the synthesis of complementary DNA (cDNA) was performed as published previously. Then, 25 ng cdNA, 0.4 µM corresponding forward and reverse primer, and SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA) were used per reaction for RT-PCR analyses. The primers were designed as published previously. Primer sequences of the respective genes are shown in the Table. The RT-PCR was performed as follows: an initial denaturation step of 95°C for 2 minutes, then 40 cycles at 95°C for 5 seconds, and at 56°C for 15 seconds, followed by an additional denaturation step for 60 seconds at 95°C. All PCR reactions were performed as duplicate and a nontemplate control (NTC) was included in all experiments. After PCR amplification, PCR products were separated by electrophoresis for 100 minutes at 150 V on a 2% agarose gel. The agarose gel was stained with GelRed (Biotium, Inc., Hayward, CA, USA) and visualized using a ChemiDoc imager (Bio-Rad). Experiments were repeated three times with different cell line passages.

**Protein Expression**

Cell lines were plated at a concentration of 2 x 10⁵ cells/mL in a 6-well plate with melanoma medium, and kept for 24 hours in 20% O2, 5% CO2, and 37°C. Melanoma medium was replaced by FBS free melanoma medium and cells were incubated again for 24 hours in 1% O2, 5% CO2, and 37°C. Afterwards, supernatants were collected to measure secreted VEGF-A, -C, and -D protein levels using human VEGF-A, -C, and -D Duoset ELISA kits (all R&D Systems, Wiesbaden, Germany). Absorbance was measured at 450 and 570 nm using an ELISA plate reader (Epoch Microplate Spectrophotometer; Biotek, Bad Friedrichshall, Germany). The quantitative analysis was performed using the software of Elisasite.com Pty Ltd. (available in the public domain at http://elisaaanalysis.com/, Melbourne, Australia). The assays were performed in triplicate and repeated three times with different cell line passages.

**Proliferation Assay**

The MT'T (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)-based proliferation assays were performed using a TACS MT'T Cell Proliferation Assay kit, (Trevigen, Gaithers-
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**TABLE.** Primer Sequences for RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence, 5′–3′</th>
<th>Product Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF-A</td>
<td>F: ACAGGTACGGGTAGAGGCAC</td>
<td>167</td>
</tr>
<tr>
<td></td>
<td>R: AAGCAGTTGACAGGAAAGCA</td>
<td></td>
</tr>
<tr>
<td>VEGF-C</td>
<td>F: GCCGTGTAATGCAAGGAAGTCC</td>
<td>163</td>
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<tr>
<td></td>
<td>R: AATATGAGGACACAGCACCAC</td>
<td></td>
</tr>
<tr>
<td>VEGF-D</td>
<td>F: CCGGCATCCATACCTGATGTT</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>R: CCCTAGCATTTCAATAGGGAG</td>
<td></td>
</tr>
</tbody>
</table>

F, forward primer sequences; R, reverse primer sequences.

W. burg, MD, USA). Blood or lymphatic endothelial cells were plated onto a 96-well plate at a concentration of 1 × 10^4 cells/well in endothelial cell medium overnight. Cells then were incubated with tumor cell-conditioned medium. After 24 hours, MTT was added resulting in intracellular formazan deposition; the reaction was stopped after 6 hours. Absorbance was measured at 570 and 700 nm with an ELISA plate reader (Epoch Microplate Spectrophotometer; Biotek). The control group (stagnant unconditioned melanoma medium without supplements) was set as 100%. The assays were performed as triplicates and the experiments were repeated five times with different cell line passages.

**Migration Assay**

Blood or lymphatic endothelial cells were seeded in a marked 96-well plate at a concentration of 3 × 10^4 cells/well in endothelial cell medium overnight. The medium was replaced by endothelial cell medium supplemented with 0.5 mM hydroxyurea (Sigma-Aldrich, Taufkirchen, Germany) to arrest cell growth and incubated for a further 24 hours. This step inhibits proliferation; thus, allowing specific testing of migration. A scratch was made using a sterile 100-μL pipette tip. In each well, a uniform straight line was drawn with the pipette tip through the confluent endothelial cell monolayer from the 6 o’clock toward the 12 o’clock position, which resulted in a uniform reproducible distance between the wound edges of the cell-free scratch. Cells were washed twice with PBS, and equal volumes of tumor cell–conditioned medium and minimal endothelial cell medium were added. At time points 0 and 24 hours, the migration was photographically documented. The analysis of the pictures was performed by ImageJ software (ImageJ 1.48v; http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). The assays were performed with 6 to 8 technical replicates and repeated three times with different cell line passages.

**Statistical Analysis**

GraphPad Prism (v. 6.00 for Windows; GraphPad Software, La Jolla, CA, USA) and SPSS (v. 21.0; IBM, Chicago, IL, USA) software was used for all statistical analyses. For each CM or UM cell line the arithmetic mean of all biological replicates was calculated. Subsequently the mean values of all three CM cell lines were grouped together and compared to grouped mean values of the three UM cell lines, to statistically assess whether the conjunctival versus uveal origin impacts prohem- and prolymphangiogenic potencies of melanoma cell lines. Statistical comparisons between CM and UM cell lines were performed using Student’s t-test if a normal distribution was assumed according to the Shapiro-Wilk test. For nonnormally distributed data the nonparametric Mann-Whitney U test was used. A P value < 0.05 was considered statistically significant.

**RESULTS**

Expression of VEGF-A, -C, and -D mRNA and Protein in CM and UM Cell Lines

We performed RT-PCR to analyze the mRNA expression of VEGF-A, -C, and -D by CM cell lines CM2005.1, CRMM1, and CRMM2, and by UM cell lines Mel270, Mel290, and OM431.

All investigated cell lines expressed VEGF-A mRNA (Fig. 1A). We also analyzed VEGF-A protein levels using a specific ELISA. The supernatants of all investigated CM and UM cell lines expressed VEGF-A protein (Fig. 1B). Cell line OM431 expressed the highest VEGF-A protein level, and Mel290 the lowest. Comparing the three CM with the three UM cell lines, the VEGF-A protein expression approached no significance (P = 0.64).

All tested CM and UM cell lines expressed VEGF-C mRNA (Fig. 1C). Interestingly, VEGF-C protein was observed only in the supernatant of the UM cell lines (P = 0.10; Fig. 1D).

The VEGF-D mRNA and VEGF-D protein were expressed in all six cell lines (Figs. 1E, 1F), and CM and UM cell lines did not differ significantly for protein expression (P = 0.42).

Impact of CM and UM Cell Lines on BEC and LEC Proliferation

Blood and lymphatic endothelial cells were cultured with CM- and UM-conditioned medium, and proliferation was measured after 24 hours (Fig. 2). All investigated cell lines showed similar proliferative effects on BECs (Fig. 2A) and LECs (Fig. 2B). No significant differences between CM and UM cell lines were detectable for BECs (P = 0.86) or LECs (P = 0.90).

Impact of CM and UM Cell Lines on BEC and LEC Migration

Blood and lymphatic endothelial cells were cultured with CM- and UM-conditioned medium, and migration was analyzed after 24 hours. Conditioned media of all CM and UM cell lines affected BEC and LEC migration to a similar extent (Fig. 3). No significant differences between CM and UM cell lines could be observed for BECs (P = 0.56; Fig. 3A) or LECs (P = 0.90; Fig. 3B).

**DISCUSSION**

Since CM spreads primarily via the lymphogenic path and UM metastasizes almost exclusively via the hematogenic path, we wanted to know whether these different metastatic patterns might be attributable to differences in the hem- and lymphangiogenic potentials of CM and UM cells. The present study shows that all CM and UM cell lines express VEGF-A, -C, and -D at the mRNA level, and VEGF-A and -D at the protein level. Protein VEGF-C is only produced by UM cell lines. The conditioned medium of CM and UM cell lines has a similar impact on proliferation and migration of BEC and LEC.

Regarding the expression of prohem- and prolymphangiogenic VEGF-A, -C, and -D, we confirmed previous studies for CM cell lines. Furthermore, we analyzed—to our knowledge for the first time—the hem- and lymphangiogenic profile of CM cell lines. The CM cell lines expressed VEGF-A and -D at mRNA and protein level, and VEGF-C only at mRNA level.
FIGURE 1. Expression levels of VEGF-A, -C, and -D in CM and UM cell lines (determined in the cell lines by RT-PCR and in culture supernatants by ELISA). All tested CM and UM cell lines expressed VEGF-A, -C, and -D mRNA, and VEGF-A and -D protein, and only UM cell lines secreted VEGF-C protein. (A) All CM and UM cell lines expressed VEGF-A mRNA. (B) Conjunctival melanoma cell lines showed similar VEGF-A protein expressions in their culture supernatants, while the UM cell lines varied greatly. (C) All investigated CM and UM cell lines expressed VEGF-C mRNA. (D) Protein VEGF-C was detectable only in the culture supernatant of UM cell lines. (E) Vascular endothelial growth factor-D mRNA expression was detected in all CM and UM cell lines. (F) Cell lines of CM did not express significantly higher amounts of VEGF-D protein than CM cell lines. NTC, nontemplate control.

FIGURE 2. Influence of CM- and UM-conditioned medium on BEC and LEC proliferation (determined in an MTT assay). Cell lines CM and UM did not show a significantly different impact on BEC (A) and LEC (B) proliferation.
In addition, our proliferation and migration assays neither showed a higher prolymphangiogenic potential of CM cell lines nor a higher prohemangiogenic potential of UM cell lines, suggesting that the type of metastatic behavior does not depend on specific expression profiles of the tumor cells. Therefore, other factors must have a role in the different routes of metastases formation, and in particular the anatomy of ocular lymphatics may have an important role. While the extraocular conjunctiva and limbus are well-endowed with lymphatic vessels, the inner eye is physiologically devoid of these vessels. The sclera seems to be a natural barrier against invasion of preexisting lymphatic vessels into the eye, despite a high intraocular expression level of VEGF-C, -D, and VEGFR-3 in UMs without extraocular extension. Due to this inaccessibility of the extraocular lymph vessel system, UMs are not able to spread via the lymphogenic route, in contrast to CMs. Only when they have grown outside the eye, may they find their way into the lymph system.

Moreover, tumor-associated hem- and lymphangiogenesis is regulated and promoted not only by tumor cells themselves, but also by cells of the tumor microenvironment, including cancer-associated fibroblasts, mesenchymal stem cells, dendritic cells, or macrophages. In fact, via recruitment of VEGF-C and VEGF-D secreting macrophages the expression of primarily prohemangiogenic VEGF-A by CM cells also might indirectly account for the lymphogenic metastatic pattern of CM. Furthermore, we did not analyze the tumor-vessel interaction in terms of regulation of adhesion molecules regulating tumor invasion into both vessel types. In addition, the extracellular matrix as well as cytokines and growth factors are involved in the process of (lymph)angiogenesis and metastasis. The cellular and noncellular components influence each other and can be influenced by the tumor cells. In general, the use of established tumor cell lines as in the present in vitro experiments has its shortcomings. Expression profiles, including prohem- and prolymphangiogenic factors, might to some degree have altered over multiple culturing passages in comparison with primary tumor cells freshly isolated from tumor tissue. Even more importantly, in vitro analyses in general, which in the present case only focus on the impact of CM or UM cell lines on BECs or LECs, can never reflect the complex multidirectional interactions within the tumor microenvironment, which among others result in metastatic spread. Therefore, further studies on the pro(lymph)angiogenic cross talk in the tumor microenvironment are needed to

**Figure 3.** Impact of CM- and UM-conditioned medium on BEC and LEC migration (determined in a scratch assay). Cell lines CM and UM did not reveal a significantly different influence on BEC (A) and LEC (B) migration. Exemplary images of BEC (C, E) and LEC (D, F) migration under CM2005.1-conditioned medium after 0 and 24 hours.
better understand the metastatic mechanism of CM and UM and fine-tune novel anti(lymph)angiogenic treatment strategies for these tumors.\textsuperscript{50,51}

In conclusion, CM cell lines do not show a higher prolymphangiogenic potential, and UM cell lines do not reveal a higher prohemangiogenic potential. Therefore, other mechanisms within the tumor microenvironment as well as the anatomical accessibility of the extraocular lymph vessels system might account for the distinct lymphangiogenic versus hemangiogenic metastatic behavior of conjunctival versus uveal melanomas.

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