Intraocular Lymphangiogenesis in Malignant Melanomas of the Ciliary Body with Extraocular Extension


PURPOSE. To analyze whether lymphatic vessels can invade the normally alymphatic eye (lymphangiogenesis) in patients with malignant melanoma of the ciliary body with extraocular extension and to correlate these findings with metastasis-free survival.

METHODS. Ten enucleated globes with the histopathologically and immunohistochemically (S-100, HMB-45, PNL-2, and Melan-A) confirmed diagnosis of malignant melanoma of the ciliary body with extraocular extension were matched with 10 globes with a ciliary body melanoma without extraocular extension, regarding tumor size (prominence, basal diameter, mitotic count, vascular networks, and patients’ age). In all 20 cases, immunohistochemistry was performed to identify lymphatic vessels by using LYVE-1 and podoplanin as specific markers for lymphatic vascular endothelium.

RESULTS. Intraocular LYVE-1+ and podoplanin+ lymphatic vessels were detected in 7 of 10 malignant melanomas of the ciliary body with extraocular extension (two of these developed a regional lymph node metastasis). Lymphatic vessels were found only at the tumor periphery directly adjacent to the sclera within the eye, more often in tumors of the epithelioid type (P = 0.017, Mann-Whitney test). Ciliary body melanomas without extrasceral extension revealed no intraocular LYVE-1+ and podoplanin+ lymphatic vessels. The presence of intraocular LYVE-1+/podoplanin+ lymphatic vessels was significantly associated with lower metastasis-free survival rates (P = 0.038, log-rank test).

CONCLUSIONS. Malignant melanomas of the ciliary body with extraocular extension show intraocular lymphatic vessels. This first evidence of lymphangiogenesis into the normally alymphatic eye may explain the increased risk of lymphatic metastasis in ciliary body melanoma with extraocular extension. Except for the extracocular conjunctiva, the human eye is physiologically devoid of lymphatic vessels, which is thought to contribute to its special immune-privileged state. However, blood and lymphatic vessels can invade the normally avascular cornea secondary to a variety of corneal diseases. The outgrowth of new from preexisting lymphatic vessels (lymphangiogenesis) has recently engaged much interest in tumor research since the outgrowth of lymphatic vessels is the initial step in lymphogenic metastasis of several tumors (e.g., malignant melanoma of the skin). Lymphangiogenesis is mediated by binding of the lymphangiogenic growth factors VEGF (vascular endothelial growth factor)-C and -D to their specific lymphatic vascular endothelial receptor, VEGF receptor 3. VEGF-C and -D can be released by a variety of tumor cells, as well as by peritumoral macrophages, thus explaining the occurrence of tumor-associated lymphangiogenesis. Although the role of peri-versus intratumoral lymphangiogenesis remains controversial, its role as a decisive risk factor for tumor metastasis is now established.

Since lymphangiogenesis seems to be an important aspect of metastasis in melanoma of the skin, its role in uveal malignant melanoma was recently evaluated. Clarijs et al. identified coexpression of both lymphangiogenic VEGF-C and its receptor VEGF receptor 3 in about half of the patients in a series of uveal melanomas, but could not, as in the normal control eyes, identify intraocular lymphatic vessels. They concluded that VEGF-C in the absence of accessible lymphatic vessels (i.e., within the closed eye) cannot induce lymphangiogenesis. Malignant melanomas with extraocular extension contrast, at least theoretically, have access to conjunctival lymphatic vessels. Since lymphatic metastasis is known to occur in patients with uveal melanoma and extraocular extension, we were interested to analyze whether lymphatic vessels can invade into the normally alymphatic eye (lymphangiogenesis) in patients with malignant melanoma of the ciliary body with extraocular extension and to correlate these findings with survival and metastasis rates.

METHODS

Patient Population and Histologic Analyses

A case-controlled, matched-pair cohort study was arranged from the archives of the Ophthalmic Pathology Laboratory of the Department of Ophthalmology in Erlangen, Germany. Of a total of 102 globes enucleated for malignant melanoma of the ciliary body between 1995 and 2007, 10 with ciliary body melanoma with extraocular extension (group 1) were closely matched with 10 eyes without extraocular tumor extension (group 2), regarding tumor size (prominence, ±0.5 mm; basal diameter, ±1.0 mm) and cell type. Further matching criteria...
Table 1. Clinical and Pathological Characteristics of 20 Patients with Malignant Melanoma of the Ciliary Body with and without Extraocular Extension

<table>
<thead>
<tr>
<th></th>
<th>Without Extraocular Extension (n = 10)</th>
<th>With Extraocular Extension (n = 10)</th>
<th>Presence of Intraocular Lymphatic Vessels (n = 7)</th>
<th>Absence of Intraocular Lymphatic Vessels (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis (y)</td>
<td>70 ± 7 (62–80)</td>
<td>68 ± 4 (59–76)</td>
<td>68 ± 5 (59–76)</td>
<td>69 ± 1 (68–70)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>4 (40)</td>
<td>5 (50)</td>
<td>4 (57)</td>
<td>1 (33)</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>6 (60)</td>
<td>5 (50)</td>
<td>3 (43)</td>
<td>2 (67)</td>
</tr>
<tr>
<td>Laterality</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right eye, n (%)</td>
<td>6 (60)</td>
<td>5 (50)</td>
<td>4 (57)</td>
<td>1 (33)</td>
</tr>
<tr>
<td>Left eye, n (%)</td>
<td>4 (40)</td>
<td>5 (50)</td>
<td>3 (43)</td>
<td>2 (67)</td>
</tr>
<tr>
<td>Apical tumor height (mm)</td>
<td>7 ± 3 (3–10)</td>
<td>7 ± 3 (3–12)</td>
<td>8 ± 2 (5–12)</td>
<td>3 ± 1 (3–4)</td>
</tr>
<tr>
<td>Largest tumor basal diameter (mm)</td>
<td>7 ± 3 (3–10)</td>
<td>7 ± 3 (3–12)</td>
<td>8 ± 2 (5–12)</td>
<td>3 ± 1 (3–4)</td>
</tr>
<tr>
<td>Tumor size (height × basal diameter; mm²)</td>
<td>71 ± 42 (12–120)</td>
<td>71 ± 46 (9–144)</td>
<td>95 ± 31 (50–144)</td>
<td>14 ± 5 (9–18)</td>
</tr>
<tr>
<td>Distance from posterior tumor border to edge of optic disc (mm)</td>
<td>15 ± 3 (12–19)</td>
<td>15 ± 4 (11–20)</td>
<td>15 ± 4 (11–20)</td>
<td>16 ± 4 (12–19)</td>
</tr>
<tr>
<td>Melanin content</td>
<td>2 (20)</td>
<td>2 (20)</td>
<td>1 (14)</td>
<td>1 (33)</td>
</tr>
<tr>
<td>Predominant tumor cell type</td>
<td>3 (30)</td>
<td>2 (20)</td>
<td>1 (14)</td>
<td>1 (33)</td>
</tr>
<tr>
<td>Mitotic count (per 40 high power fields)</td>
<td>3 (30)</td>
<td>3 (30)</td>
<td>3 (43)</td>
<td>1 (33)</td>
</tr>
<tr>
<td>Vascular networks</td>
<td>3 (30)</td>
<td>5 (50)</td>
<td>5 (71)</td>
<td>1 (33)</td>
</tr>
<tr>
<td>Absent, n (%)</td>
<td>7 (70)</td>
<td>5 (50)</td>
<td>2 (29)</td>
<td>3 (100)</td>
</tr>
</tbody>
</table>

 included distance from the optic disc (±1.0 mm), melanin content, mitotic count (±0.5 mitoses per 40 high power fields), presence of vascular networks, and patients’ ages. Patients with malignant melanoma of the ciliary body with (group 1) and without extraocular extension (group 2) showed comparable distributions of clinical and pathologic tumor characteristics (Table 1). In none of the patients did general physical examination and oncological work-up at the time of diagnosis reveal any evidence of another primary tumor, hematogenic metastasis, or lymph node metastasis. As a control tissue, we used one normal eye obtained by eyelid-sparing orbital exenteration for a mucoepidermoid carcinoma of the orbit. All enucleated globes were fixed in a buffered 4% formaldehyde solution, dehydrated, and embedded in paraffin. Multiple serial sections of the entire tumor area cut at 5 μm were stained with hematoxylin-eosin, periodic acid-Schiff, S-100 (rabbit, 1:400; Dako, Hamburg, Germany), anti-melanosome HMB-45 (mouse, 1:400; Dako), anti-melanoma PNL-2 (mouse, ready-to-use; Dako), and melan-A (mouse, 1:50; Dako) and analyzed by three independent investigators by microscopy (Axiophot; Carl Zeiss Meditec, Oberkochen, Germany) to confirm the diagnosis histopathologically as well as immunohistochemically.

### Immunohistochemistry

To identify lymphatic vessels, immunohistochemistry was performed in all 20 cases as described previously. Therefore, a polyclonal antibody against the human lymphatic vascular endothelial specific hyaluronic acid receptor LYVE-1 (rabbit, 1:100; ACRIS, Herford, Germany) and a monoclonal antibody against the human lymphatic vascular endothelial specific glycoprotein podoplanin D2-40 (mouse, 1:40; AbD Scrotec, Kidlington, Oxford, UK) were used. Serial sections were evaluated for evidence of intraocular LYVE-1⁺ and podoplanin⁺ lymphatic vessels by three independent investigators in a masked fashion (Axiophot microscope; Carl Zeiss Meditec) after digital documentation. Main outcome measures included the presence, localization and highest number of LYVE-1⁺/podoplanin⁺ lymphatic vessels (per section) within the eye as well as in the extraocular part of the tumor.

### Confocal Laser Scanning Microscopy

As a second method of identifying LYVE-1⁺ and podoplanin immunoreactive structures, fluorescence immunohistochemistry was applied in paraffin-embedded sections. After preincubation of the slides with 10% normal goat serum (Dako) in typical order, LYVE-1 and podoplanin...
immunoreactivity was visualized by corresponding Alexa568 goat anti rabbit- and Alexa488 goat anti mouse IgG-tagged antisera (1:1000; Invitrogen, Karlsruhe, Germany). Slides were embedded in TBS-glycerol (1:1 at pH 8.6). Negative controls were performed by omission of the primary antibodies during incubation and resulted in no staining. Further, the tissue was treated with a commercially available negative control reagent (Dako), which resulted in no staining. Podoplanin was replaced with a monoclonal antibody of the same isotype with an established binding pattern and revealed the absence of lymphatic vessels. To avoid misinterpretation in the masked studies, a color coding (LYVE-1, red; podoplanin, green) was maintained throughout the experiments and the report. To regain antigenicity for immunohistochemistry in paraffin-embedded sections, different pretreatment protocols were necessary for the LYVE-1 (microwave irradiation, 600 W, 2 × 3 minutes at pH 6.0) and podoplanin antiserum (no pretreatment), respectively. However, these different protocols resulted in an incompatibility of both antisera at the same time, thus obviated double immunohistochemistry on same sections. To circumvent this problem, alternating serial sections were prepared and the congruent areas were compared.

To document single- and double-label immunohistochemistry, we used a confocal laser scanning microscope equipped with a digital camera an krypton argon laser (MRC 1000; Bio-Rad, Munich, Germany) with to a Diaphot 300; Nikon, Düsseldorf, Germany, with a krypton-argon laser; ALC, Salt Lake City, UT, and a ×60 oil immersion objective lens with numeric aperture 1.4; Nikon). Sections were imaged in the single optical section mode with the appropriate filter settings for Alexa568 (568 nm excitation, filter 605DF32; channel 1, coded red) and Alexa488 (488 nm excitation, filter 522DF32; channel 2, coded green). Colocalization of the same structures in channels 1 and 2 resulted in a yellow mixed color. Since background fluorescence is discrete in channel 1 with the excitation of 568 nm used to detect LYVE-1 immunohistochemistry, both channels were electronically overlaid, which resulted in a better signal-to-noise ratio.

**Transmission Electron Microscopy**

Transmission electron microscopy was performed in one tumorous eye with extraocular extension after re-embedding of the paraffin-embedded tissue in epoxy resin, according to standard protocols. Semithin sections were stained with toluidine blue, and the ultrathin sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (EM 906E; Carl Zeiss NTS GmbH).

**Clinical Data**

All clinical files were reviewed retrospectively with special regard to tumor staging. In a standardized telephone interview in 2008, all patients and/or their physicians were asked about survival and new onset of distant metastases or lymph node metastasis. This retrospective, nonrandomized, clinicopathologic single-center study was performed in conformance with the tenets of the Declaration of Helsinki.
Institutional Review Board/Ethics Committee approval was not required in this instance.

Mean follow-up time was similar in patients with ciliary body melanoma with extraocular extension (group 1: 80 ± 27 months; range, 22–122 months) and patients without extraocular melanoma growth (group 2: 87 ± 25 months; range, 60–142 months).

In five patients in group 1 and two patients in group 2, distant metastases developed within a mean interval of 28 ± 11 months (range, 14–42 months) after enucleation. In all seven cases, distant metastatic spread occurred in the liver and, in two patients, in the lung.

Simultaneously, regional lymph node metastases were detected in two patients in group 1: one patient had clinically and histologically proven cervical and thoracic lymph node metastases 18 months after enucleation and the other had only clinically diagnosed, cervical lymph node metastases 2 years after surgery. None of the patients in group 2 showed clinical evidence of lymph node metastases.

In group 1, five patients died of melanoma, and in group 2, two patients died of melanoma and one of cardiovascular disease. The 5-year cumulative metastasis-free survival rate was not significantly different in patients with (45% ± 17%) versus without (80% ± 13%) extraocular extension (P = 0.079, log-rank test).

Statistical Analyses
Commercial software was used for all statistical analyses (SPSS ver. 15.0 for Windows; SPSS, Inc., Chicago, IL). Comparisons between groups or variables were performed with the nonparametric Mann-Whitney test, the Pearson’s χ² test, and the Spearman correlation coefficient. Survival rates ± SE were determined according to the Kaplan-Meier method and compared by using the log-rank test. P < 0.05 was considered statistically significant.

RESULTS
Detection of Peritumoral Intraocular Lymphatic Vessels
To test our hypothesis that lymphatic vessels can proliferate into the normally alymphatic eye in patients with malignant

**FIGURE 2.** Top: Sketch of the anterior half of the eye showing sites investigated in the images in (A) to (C). Cor, cornea; AC, anterior chamber; L, lens; TU, tumor; Sc, sclera. (A, A’) Extraocular location of the tumor: LYVE-1 immunoreactivity (A, arrowheads) and podoplanin (A’, bright green) was detected on the endothelium of lymphatic vessels, but was absent on vascular endothelium (as recognized by yellow autofluorescent erythrocytes in A and arrows in A’). (B, B’) Transition site of the tumor from extraocular to intraocular: LYVE-1 immunoreactivity (B, arrowheads) and podoplanin (B’, bright green) was detected on the endothelium of lymphatic vessels, but was absent on vascular endothelium (as shown by the yellow autofluorescent erythrocytes in B and arrows in B’). (**) Conjunctival margin. (C, C’) Intraocular location of the tumor: LYVE-1 immunoreactivity (C, arrowheads) and podoplanin (C’, bright green) was detected on the endothelium of lymphatic vessels.
melanomas of the ciliary body with extraocular extension, we compared ciliary body melanomas with (group 1) and without extraocular extension (group 2) regarding the immunohistochemical identification of intraocular lymphatic vessels.

Intraocular LYVE-1+ and podoplanin+ lymphatic vessels were detected in 7 of 10 malignant melanomas of the ciliary body with extraocular extension (group 1). Lymphatic vessels were found only at the tumor periphery directly adjacent to the sclera within the eye, whereas the central aspects of the intraocular tumor showed no LYVE-1+ or podoplanin+ lymphatic vessels (Fig. 1). The number of intraocular lymphatic vessels averaged 20 ± 7 (range, 13–30) per section.

In the extraocular tumor component, LYVE-1+ and podoplanin+ lymphatic vessels were observed in all 10 melanomas with extraocular tumor extension (group 1), but only within the tumor periphery adjacent to the conjunctiva and not within the central aspects of the extraocular tumor part (Fig. 1).

Ciliary body melanomas without extrascleral extension (group 2) revealed neither intraocular LYVE-1+ nor podoplanin+ lymphatic vessels within the ciliary body tumor or its vicinity.

Intraocular lymphatic vessels were seen only in malignant melanoma of the ciliary body with extraocular extension, but not in melanomas without extrascleral extension (P < 0.001, Pearson’s χ² test).

In single optical sections of the confocal microscope, LYVE-1 and podoplanin immunoreactivity was detected on lymphatic endothelium, but was absent on vascular endothelium in a tumorous eye with extraocular extension (Fig. 2). In contrast, LYVE-1 and podoplanin immunoreactivity was absent in our control eye devoid of ciliary body melanoma (Fig. 3).

Transmission electron microscopy revealed intraocular lymphatic vessels surrounded by scleral collagen fibrils underlying the tumor base in an eye with a malignant melanoma of the ciliary body and extraocular tumor extension (Fig. 4).

Intraocular Lymphangiogenesis and Tumor Size, Cell Type, and Metastasis-free Survival

Studying the association between intraocular lymphangiogenesis and tumor size, tumor cell type, and metastasis-free survival, the subsequent statistical analyses were performed only on the 10 patients with extraocular tumor growth (group 1), to avoid introducing a bias by adding 10 probably negative cases of patients with ciliary body melanoma without extraocular tumor extension (group 2).

Tumor Size. In group 1, a higher number of intraocular LYVE-1+/podoplanin+ lymphatic vessels per section correlated significantly with a larger size of the intraocular (Spearman r = 0.928; P < 0.001) as well as extraocular tumor component (Spearman r = 0.897; P < 0.001; Fig. 5A).

Tumor Cell Type. Significantly more intraocular LYVE-1+/podoplanin+ lymphatic vessels per section were detectable in epitheloid-cell than in mixed-cell or spindle-cell types of malignant melanomas of the ciliary body with extraocular extension (P = 0.017, Mann-Whitney test; Fig. 5B).

Metastasis-free Survival. Patients in group 1 who had distant metastases revealed a significantly higher number of intraocular LYVE-1+/podoplanin+ lymphatic vessels per section than did patients without metastatic spread (P = 0.032, Mann-Whitney test). Regional lymph node metastasis was associated with more intraocular LYVE-1+/podoplanin+ lymphatic vessels per section that failed to reach statistical significance (P = 0.064, Mann-Whitney test).

The 5-year cumulative metastasis-free survival rate was 34% (SE 20%) for ciliary body melanomas with extraocular extension disclosing intraocular LYVE-1+/podoplanin+ lymphatic vessels, and 100% (SE 0%) for those without intraocular lymphangiogenesis. Absence of intraocular LYVE-1+/podoplanin+ lymphatic vessels was significantly associated with higher metastasis-free survival rates (P = 0.038, log-rank test; Fig. 6).

DISCUSSION

The present study reveals two novel important findings: first, that intraocular LYVE-1+/podoplanin+ lymphatic vessels can
be detected in malignant melanomas of the ciliary body with extraocular extension. This is the first evidence of secondary lymphangiogenesis into the normally alymphatic intraocular space. Second, the presence of intraocular LYVE-1+/podoplanin+ lymphatic vessels seems to be associated with lower metastasis-free survival, thus establishing immunohistochemical detection of intraocular, tumor-associated LYVE-1+/podoplanin+ lymphatic vessels as a probable novel prognostic indicator in malignant melanomas of the ciliary body with extraocular extension.

Although the concept of tumor-associated lymphangiogenesis is now well established for several extraocular tumors, it was thought that VEGF-C release by intraocular melanomas could not induce lymphangiogenesis due to lack of accessible preexisting lymphatic vessels within the eye.12 This is in contrast to the situation within the normally also alymphatic cornea, which has ready access to preexisting limbal lymphatic vessels from which secondary lymphangiogenesis can start. Therefore, several inflammatory diseases of the cornea can induce corneal lymphangiogenesis by outgrowth of new lym-

**FIGURE 4.** Transmission electron micrograph of a lymphatic vessel surrounded by scleral collagen fibrils underlying the tumor base in an eye with malignant melanoma of the ciliary body and extraocular tumor extension. The weak and discontinuous basement membrane of the endothelial lining (arrows), a typical feature of lymphatic vessels, was clearly visible despite the poor quality of structural preservation due to re-embedding of the paraffin-embedded tissue in epoxy resin. co, collagen; en, endothelium; lu, vessel lumen. Scale bars: (A) 5 μm; (B, C) 1 μm.

**FIGURE 5.** Tumor size and tumor cell type correlate with intraocular lymphangiogenesis in ciliary body melanomas with extraocular extension. (A) Larger tumors showed a higher number of intraocular tumor-associated LYVE-1+/podoplanin+ lymphatic vessels per section (Spearman r = 0.928; P < 0.001). The line denotes the linear regression. (B) Higher intraocular tumor-associated LYVE-1+/podoplanin+ lymphatic vessel number per section was observed in epithelioid than in mixed and spindle cell types (P = 0.017, Mann-Whitney test).
Although we favor the concept of attracting conjunctival lymphatic vessels into the eye (secondary intraocular lymphangiogenesis), we cannot rule out the remote possibility of a de novo formation of intraocular lymphatics, since it has recently been shown that Prox-1+ macrophage subpopulations can differentiate into lymphatic vascular endothelium. Nonetheless, the localization next to the sclera and the occurrence only in the presence of the scleral defect suggest secondary invasion of conjunctival lymphatic vessels. Regarding the major source of VEGF-C, which attracts extraocular lymphatic vessels to the eye, the tumor cells themselves as well as peritumoral macrophages are potential candidates.

The uveoscleral outflow pathway has been compared to lymphatic drainage, and recently a new physiological uveolympathic outflow pathway was suggested for the human and sheep ciliary body (Gupta N et al. IOVS 2008;ARVO E-Abstract 2879). However, the absence of lymphatic markers in our control tissues indicates that the normal eye is thought to be devoid of true lymphatic vessels. Our findings provide the first evidence that "secondary lymphangiogenesis" into such an alymphatic organ can occur. A similar finding was obtained for the cornea, but this does not actually involve ingrowth of lymphatics into the intraocular space.

It should be emphasized that double immunohistochemistry on paraffin-embedded sections to detect LYVE-1 and podoplanin was not feasible, despite various protocols, which is in contrast to results obtained in cryosections not taken from paraffin-embedded sections. However, comparison of serial sections revealed the presence of both markers on the lymphatic endothelium, but clearly showed their absence in vascular endothelium and thus demonstrated the specificity of both markers for lymphatic vessels in the ciliary body melanoma with extraocular extension investigated in our study.

Our findings also established the presence of intraocular, tumor-associated LYVE-1+/podoplanin+ lymphatic vessels as a probable novel prognostic indicator for metastasis of ciliary body melanomas with extraocular extension. However, these results are limited to a small number of patients and should be verified in larger prospective series. Although the precise mechanism is unknown, probable reasons for this association include the known correlation between higher metastasis rates and larger tumors as well as epithelioid tumor cell types, both disclosing more intraocular lymphatic vessels, a role of VEGF-C in mediation of metastasis and changes in the ocular microenvironment due to scleral defects associated with a higher risk of metastasis.

The data in the present study suggest a potential for novel antilymphangiogenic therapies in patients with uveal malignant melanomas with extraocular extension, to prevent spread of the tumor to regional lymph nodes via the lymphatic vessels.

In conclusion, malignant melanomas of the ciliary body with extraocular extension show intraocular lymphatic vessels that deserve attention. This first evidence of lymphangiogenesis in the normally alymphatic eye may explain the increased risk of regional lymphatic metastasis in ciliary body melanoma with extraocular extension.

References


