Intraocular Lymphatics in Ciliary Body Melanomas With Extraocular Extension

Functional for Lymphatic Spread?

Ludwig M. Heindl, MD; Tanja N. Hofmann, MD; Falk Schrödl, MD; Leonard M. Holbach, MD; Friedrich E. Kruse, MD; Claus Cursiefen, MD

Objective: To assess the functional significance of intraocular tumor–associated lymphatic vessels in ciliary body melanomas with extraocular extension.

Methods: Twelve consecutive patients enucleated for a malignant melanoma of the ciliary body with extraocular extension and immunohistochemical presence of intraocular LYVE-1–positive and podoplanin-positive lymphatic vessels were examined for proliferation status and tumor invasion into tumor-associated lymphatics. Proliferating lymphatic vessels were identified using LYVE-1 and podoplanin as specific lymphatic endothelial markers and Ki-67 as the proliferation marker. Tumor invasion into lymphatic vessels was assessed using Melan-A as the melanoma marker. Kaplan-Meier analyses of survival and metastasis were performed.

Results: Intraocular proliferating lymphatic vessels were detected in all 12 ciliary body melanomas with extraocular extension. The ratio of proliferating lymphatics was significantly higher in the intraocular vs extraocular tumor compartment (P < .001). Extraocular lymphatic invasion by tumor cells was observed in 5 patients (42%), intraocular lymphatic invasion in 4 (33%), and synchronous intraocular and extraocular lymphatic invasion in 3 (25%). Detection of melanoma cells in intraocular and extraocular lymphatic vessels was significantly associated with higher risks of lymphatic spread (P < .001) and lower metastasis-free survival rates (P = .03).

Conclusions: Intraocular tumor–associated lymphatic vessels contain proliferating endothelial cells and can be invaded by cancer cells in ciliary body melanomas with extraocular extension. Lymphatic invasion by tumor cells seems to be associated with an increased risk of lymphatic spread and mortality in these affected patients.
Lignant melanomas of the ciliary body can attract extraocular lymphatics into the normally alymphatic eye when extraocular tumor extension provides routes of entry through the sclera. This intraocular tumor–associated lymphangiogenesis was correlated with a poorer survival prognosis.

The objective of the present study was to assess the functional significance of intraocular tumor–associated lymphatic vessels in ciliary body melanomas with extraocular extension. Therefore, we analyzed whether these intraocular lymphatics are actively proliferating and can be invaded by tumor cells, and we correlated these findings with metastasis and survival rates.

**METHODS**

Between January 1, 1995, and December 31, 2007, 102 consecutive patients underwent primary enucleation for a malignant melanoma of the ciliary body in the Department of Ophthalmology, University Eye Hospital, Friedrich-Alexander University Erlangen-Nürnberg, Germany. All enucleated globes were fixed in a buffered formaldehyde-glutaraldehyde solution, 4%, dehydrated, and embedded in paraffin.

Multiple serial sections of the entire tumor cut at 5 µm were stained with hematoxylin-eosin, periodic acid–Schiff, S-100 protein (rabbit, 1:400) (Dako, Hamburg, Germany), antimelanosome HMB-45 (mouse, 1:400) (Dako), antimelanoma PNL-2 (mouse, ready-to-use) (Dako), and Melan-A (mouse, 1:50) (Dako) and were analyzed by 3 independent investigators (L.M.H., T.N.H., and C.C.) using an epifluorescence microscope to detect extrascleral tumor extension.

In ciliary body melanomas with proven extraocular extension, lymphatic vessels were identified immunohistochemically using a polyclonal antibody against the human lymphatic vascular endothelial-specific hyaluronic acid receptor LYVE-1 and a monoclonal antibody against the human lymphatic vascular endothelial-specific glycoprotein podoplanin D2-40 as described previously. Serial sections were evaluated for evidence of intraocular LYVE-1–positive and podoplanin-positive lymphatic vessels by 3 independent investigators in a masked manner using an epifluorescence microscope. The presence of intraocular lymphatic vessels was defined as detection of at least 5 LYVE-1–positive and podoplanin-positive lymphatic vessels per section with an erythrocyte-free lumen located below the inner scleral layers in the intraocular tumor compartment.

The inclusion criteria for this retrospective, nonrandomized, single-center study were (1) histologic diagnosis of a ciliary body melanoma with extraocular extension, (2) presence of intraocular LYVE-1–positive and podoplanin-positive lymphatic vessels, and (3) presence of at least 5 LYVE-1–positive and podoplanin-positive lymphatic vessels per section.

**Table. Clinicopathologic Characteristics of 12 Patients With Ciliary Body Melanomas With Extraocular Extension in the Presence or Absence of Tumor Invasion Into Intraocular Lymphatic Vessels**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Tumor Invasion Into Intraocular Lymphatics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Present (n=4)</td>
</tr>
<tr>
<td>Age at diagnosis, mean (SD) [range], y</td>
<td>70 (5) [65-76]</td>
</tr>
<tr>
<td>Male sex, No. (%)</td>
<td>2 (50)</td>
</tr>
<tr>
<td>Right eye laterality, No. (%)</td>
<td>2 (50)</td>
</tr>
<tr>
<td>Apical tumor height, mean (SD) [range], mm</td>
<td>9 (2) [7-12]</td>
</tr>
<tr>
<td>Apical tumor height, No. (%)</td>
<td></td>
</tr>
<tr>
<td>2.0-5.0 mm</td>
<td>0</td>
</tr>
<tr>
<td>5.1-8.0 mm</td>
<td>3 (75)</td>
</tr>
<tr>
<td>&gt;8.0 mm</td>
<td>1 (25)</td>
</tr>
<tr>
<td>Largest basal tumor diameter, mean (SD) [range], mm</td>
<td>12 (3) [8-14]</td>
</tr>
<tr>
<td>Largest basal tumor diameter, No. (%)</td>
<td></td>
</tr>
<tr>
<td>3.0-7.0 mm</td>
<td>0</td>
</tr>
<tr>
<td>7.1-10.0 mm</td>
<td>1 (25)</td>
</tr>
<tr>
<td>10.1-15.0 mm</td>
<td>3 (75)</td>
</tr>
<tr>
<td>Tumor size, mean (SD) [range], mm² (height × basal diameter)</td>
<td>102 (37) [56-144]</td>
</tr>
<tr>
<td>Distance from posterior tumor border to edge of optic disc, mean (SD) [range], mm</td>
<td>14 (4) [11-20]</td>
</tr>
<tr>
<td>Tumor pigmentation, No. (%)</td>
<td></td>
</tr>
<tr>
<td>Amelanotic to weak</td>
<td>0</td>
</tr>
<tr>
<td>Moderate</td>
<td>2 (50)</td>
</tr>
<tr>
<td>Strong</td>
<td>2 (50)</td>
</tr>
<tr>
<td>Predominant tumor cell type, No. (%)</td>
<td></td>
</tr>
<tr>
<td>Epithelioid</td>
<td>2 (50)</td>
</tr>
<tr>
<td>Mixed</td>
<td>1 (25)</td>
</tr>
<tr>
<td>Spindle</td>
<td>1 (25)</td>
</tr>
<tr>
<td>Mitotic rate per 40 high-power fields, mean (SD) [range]</td>
<td>5 (2) [3-7]</td>
</tr>
<tr>
<td>Microvascular patterns, No. (%)</td>
<td></td>
</tr>
<tr>
<td>No loops</td>
<td>1 (25)</td>
</tr>
<tr>
<td>Loops only</td>
<td>1 (25)</td>
</tr>
<tr>
<td>Networks</td>
<td>2 (50)</td>
</tr>
<tr>
<td>TNM classification, No. (%)</td>
<td></td>
</tr>
<tr>
<td>T2dN0M0</td>
<td>2 (50)</td>
</tr>
<tr>
<td>T3dN0M0</td>
<td>2 (50)</td>
</tr>
</tbody>
</table>

©2010 American Medical Association. All rights reserved.
phatic vessels, and (3) minimum follow-up of 12 months after enucleation. Twelve patients fulfilled these criteria and were included in the present clinicopathologic study performed in conformance with the tenets of the Declaration of Helsinki. Institutional review board or ethics committee approval was not required.

**PATIENT POPULATION AND CLINICAL DATA**

The 12 included patients (7 women and 5 men) had a mean (SD) age of 69 (5) years (age range, 59–76 years). Their clinical files were reviewed retrospectively with special regard to tumor staging. According to the 2009 TNM staging system, 7 tumors (58%) were classified as T2d and 5 (42%) as T3d. None of the patients during general physical examination or oncologic workup at the time of diagnosis revealed evidence of another primary tumor, hematogenic metastasis, or lymph node metastasis (Table).

In a standardized telephone interview in 2008, all the patients and their physicians were asked for data on survival and new onset of distant metastasis or lymph node metastasis. Mean (SD) follow-up was 89 (30) months (range, 22–130 months). Nine patients (75%) developed distant metastases in a mean (SD) of 41 (31) months (range, 15–121 months) after enucleation. In all 9 patients, distant metastatic spread occurred into the liver, and in 2 patients the lung was also involved. Simultaneously, regional lymph node metastasis was detected in 3 patients (25%): 1 patient developed clinically (ultrasonography and magnetic resonance imaging) and histologically proven ipsilateral cervical and thoracic lymph node metastases 18 months after enucleation and 2 patients developed only clinically (ultrasonography and magnetic resonance imaging) diagnosed ipsilateral cervical lymph node metastases 2 and 3 years postoperatively. Of all 12 patients, 9 (75%) died of melanoma. Mean (SD) 5-year cumulative metastasis-free survival was 28% (14%).

**IMMUNOHISTOCHEMICAL DETECTION OF THE PROLIFERATION STATUS OF LYMPHATIC VESSELS**

To identify lymphatic vessels as active and proliferating, paraaffin-embedded sections from all 12 patients were double stained using (1) a polyclonal antibody against the human lymphatic vascular endothelial-specific hyaluronan receptor LYVE-1 (rabbit, 1:100) (ACRIS) and a monoclonal antibody against Ki-67 (mouse, 1:100) (AbD Serotec) as a specific marker for proliferating cells30 and (2) a monoclonal antibody against the human lymphatic vascular endothelial-specific glycoprotein podoplanin D2-40 (mouse, 1:40) (AbD Serotec) and a polyclonal antibody against Melan-A (rabbit, 1:100) (Zytomed, Berlin, Germany). Antibodies against LYVE-1 and podoplanin did not tolerate any bleaching procedures of the sections.

Alternating serial sections were analyzed for evidence of tumor invasion into lymphatic vessels by 3 independent investigators (L.M.H., T.N.H., and C.C.) in a masked manner using an epifluorescence microscope after digital documentation. Tumor invasion into lymphatic vessels was considered to be present if at least 1 Melan-A–positive tumor cell cluster was clearly visible inside an LYVE-1–positive and podoplanin–positive lymphatic vessel. Main outcome measures included the presence of intraocular and extraocular lymphatic invasion by melanoma cells.

To further confirm tumor invasion into lymphatic vessels, double-fluorescence immunohistolochemical analysis against podoplanin and Melan-A was applied in paraffin-embedded sections. After preincubation of the slides with normal goat serum, 10% (Dako) in typical order, podoplanin and Melan-A immunoreactivity was visualized by corresponding Alexa568 goat anti-mouse and Alexa488 goat anti–rabbit IgG-tagged antibodies (1:1000) (Invitrogen, Karlsruhe, Germany), respectively. Slides were covered with Tris-buffered saline–glycerol mounting medium (1:1 at pH 8.6). Negative controls were performed by omission of the primary antibodies during incubation and resulted in no staining. Furthermore, the tissue was treated with a commercially available negative control reagent (Dako), which resulted in no staining. To avoid misinterpretation in the masked studies, color coding (podoplanin, red; Melan-A, green) was maintained throughout the experiments and the report. For documentation, an inverted fluorescence microscope was used (Axio Observer Z1; Carl Zeiss Micro Imaging, Göttingen, Germany) with ×20 dry and ×40 and ×63 oil immersion objective lenses (numeric apertures of 0.8, 1.3, and 1.4, respectively) connected to a confocal laser scanning unit (LSM 710; Zeiss). Sections were imaged using the single optical section mode with the appropriate filter settings for Alexa568 (excitation, 568 nm; Zeiss filter block, 43; channel 1, coded red) and Alexa488 (excitation, 488 nm; Zeiss filter block, 38; channel 2, coded green).

**STATISTICAL ANALYSIS**

Commercial software was used for all statistical analyses (SPSS version 15.0 for Windows; SPSS Inc, Chicago, Illinois). Comparisons of the ratio of proliferating lymphatic vessels and the presence of lymphatic invasion by melanoma cells with the other clinicopathologic variables were performed using the nonparametric Mann-Whitney test, the nonparametric Kruskal-Wallis test, and the Fisher exact test. Survival and metastasis rates with standard errors were determined according to the Kaplan-Meier method and were compared using the log-rank test. \( P < .05 \) was considered statistically significant.

**RESULTS**

In sections of normal conjunctiva, numerous large LYVE-1–positive and podoplanin–positive lymphatic vessels were detected in the subepithelial layer, clearly distinguished from adjacent erythrocyte-filled blood vessels, which did not stain for LYVE-1 or podoplanin (Figure 1). In all
12 ciliary body melanomas with extraocular extension, LYVE-1–positive and podoplanin-positive lymphatic vessels were observed in the extraocular tumor component but only in the tumor periphery adjacent to the conjunctiva and not in the central aspects of the extraocular tumor part (Figure 2A and B).

Intraocular LYVE-1–positive and podoplanin-positive lymphatic vessels were found in all 12 patients only at the tumor periphery directly adjacent to the sclera in the eye (Figure 2F and G). The central aspects of the intraocular tumor mass showed no LYVE-1–positive or podoplanin-positive lymphatic vessels. The intraocular lymphatic vessels had mostly a distinctive reticular architecture with numerous tiny lumina (Figure 2F and G) that differed markedly from the larger and more dilated architecture of lymphatic vessels found in the periphery of the extraocular tumor component (Figure 2A and B) or in normal conjunctival tissue (Figure 1A and B). The mean (SD) number of intraocular LYVE-1–positive and podoplanin-positive lymphatic vessels was 19 (7) (range, 11-30) per section.

**Proliferation Status of Lymphatic Vessels**

Using LYVE-1/Ki-67 and podoplanin/Ki-67 double staining, multiple lymphatic endothelial cells showing nuclear Ki-67 positivity identified tumor-associated lymphatic vessels as active and proliferating (Figure 2H and I). In the 12 enucleated globes, proliferating lymphatic vessels were detected in the intraocular and extraocular tumor components. A mean (SD) of 87% (7%) (range, 73%-95%) of intraocular lymphatic vessels and 45% (7%) (range, 33%-56%) of extraocular lymphatic vessels were proliferating (P < .001).

The intraocular and extraocular ratios of proliferating lymphatics were not significantly associated with age, sex, tumor size, distance to optic disc, tumor pigmentation, tumor cell type, mitotic rate, microvascular patterns, and TNM classification. In the normal conjunctival specimens analyzed, no dividing Ki-67–positive nuclei were observed in the lymph vessel endothelia, suggesting a nonproliferating status of normal preexisting conjunctival lymphatics (Figure 1C and D).
TUMOR INVASION INTO LYMPHATIC VESSELS

LYVE-1/Melan-A and podoplanin/Melan-A double staining revealed tumor cell clusters invading extraocular lymphatic vessels in 5 patients (42%) (Figure 2C and D). In intraocular lymphatics, melanoma cell clusters were detected in 4 patients (33%) (Figure 2J and K). Tumor invasion into intraocular and extraocular lymphatic vessels was seen in 3 patients (25%).

Using confocal laser scanning microscopy, Melan-A immunoreactive tumor invasion into podoplanin immunoreactive lymphatic vessels was confirmed in the intraocular and extraocular tumor component (Figure 3). No significant association was noted between the immunohistochemical detection of tumor invasion into intraocular or extraocular lymphatics and age, sex, tumor size, distance to the optic disc, tumor pigmentation, tumor cell type, mitotic rate, microvascular patterns, and TNM classification (Table). All 3 patients with detectable tumor cells in intraocular and extraocular lymphatics developed lymphatic spread and died during follow-up.

Using Kaplan-Meier analysis, a higher risk of regional lymph node metastasis was significantly associated with the detection of extraocular lymphatic invasion ($P = .03$) (Figure 4A), intraocular lymphatic invasion ($P = .006$) (Figure 4C), and synchronous intraocular and extraocular lymphatic invasion ($P < .001$) (Figure 4E). The presence of synchronous invasion was a stronger prognostic predictor of lymphatic spread than was intraocular or extraocular lymphatic invasion alone.

Regarding melanoma-specific mortality, the detectable tumor invasion into intraocular and extraocular lymphatic vessels was significantly associated with lower metastasis-free survival rates ($P = .03$) (Figure 4F). The association between intraocular ($P = .24$) (Figure 4D) or extraocular ($P = .77$) (Figure 4B) lymphatic invasion alone did not reach significance.

The present study reveals 2 novel findings: first, most of the intraocular tumor-associated lymphatic vessels in cili-
ary body melanomas with extraocular extension are proliferating, thus constituting considerable evidence for the presence of active lymphangiogenesis in this entity, and second, intraocular lymphatics can be the target of cancer cell invasion. This lymphatic invasion by tumor cells seems to be associated with an increased risk of lymphatic spread and mortality in these patients, supporting the concept that intraocular tumor–associated lymphatic vessels can be of functional significance in ciliary body melanomas with extraocular extension.

Apart from the extraocular conjunctiva, the human eye is physiologically devoid of lymphatic vessels. Even the normal human choroid, endowed with numerous LYVE-1–positive macrophages, does not contain typical lymphatic vessels. Recently, a new physiologic uveolymphatic outflow pathway was suggested for the human and sheep ciliary body. However, the absence of LYVE-1–positive/podoplanin-positive lymphatic vessels with an erythrocyte-free lumen in the ciliary body region of human control eyes devoid of ciliary body melanoma and eyes with ciliary body melanoma without extraocular extension indicates that the normal eye is thought to be physiologically devoid of true lymphatic vessels. Owing to this lack of accessible preexisting lymphatic vessels in the eye, it was thought that VEGF-C release by intraocular melanomas could not induce lymphangiogenesis. This is in contrast to the situation in the normally also alymphatic cornea, which has ready access to preexisting limbal lymphatic vessels.

Figure 4. Kaplan-Meier time-to-event curves of lymphatic spread (A, C, and E) and metastasis-free survival (B, D, and F) for patients with ciliary body melanomas with extraocular extension according to detection of extraocular (A and B), intraocular (C and D), and synchronous intraocular and extraocular (E and F) lymphatic invasion by tumor cells.
phatic vessels from which secondary lymphangiogenesis can start in several inflammatory diseases of the cornea.29 The present data suggest that in a similar manner, preexisting conjunctival lymphatic vessels can outgrow and proliferate into the eye when extracocular tumor extension provides routes of entry through the sclera, which otherwise seems to be a natural barrier against the invasion of lymphatic vessels.

The hypothesis that the intraocular lymphatics that we detected are newly proliferating rather than trapped preexisting or hyperplastic lymph vessels is supported by the following observations. First, unlike lymphatic vessels of the normal conjunctiva, intraocular tumor–associated lymphatics contained Ki-67–positive, newly dividing nuclei in the lymphatic endothelial cells. Second, these intraocular lymphatic vessels were mostly located adjacent to the sclera in the eye and occurred only in the presence of the scleral defect. Three possibilities can be discussed as an origin of these proliferating lymphatic vessels: (1) conjunctival lymphatics proliferate and invade the melanoma, (2) melanoma lymphatics proliferate and invade the conjunctiva, and (3) a mixture of (1) and (2). At this stage, we cannot rule out the remote possibility of a de novo formation of intraocular lymphatics because it has recently been shown that Prox-1–positive macrophage subpopulations can differentiate into lymphatic vascular endothelium.20 Nevertheless, in view of the localization next to the sclera and the occurrence only in case of a scleral defect, we favor the concept of attracting conjunctival lymphatic vessels into the eye (secondary intraocular lymphangiogenesis). This kind of active “lymphangiotaxis” may be the result of increased levels of prolymphangiogenic growth factors, such as VEGF-C,25 and decreased levels of antilymphangiogenic inhibitors owing to the scleral weakness. However, we did not observe distinct connections between the preexisting conjunctival lymphatics and the proliferating intratumoral lymphatics, but the immediate vicinity suggests their likely existence.

The main significance of proliferating intratumoral lymphatic vessels is that they could provide a possible route for the lymphatic spread of ciliary body melanomas with extraocular extension into the regional lymph nodes. Indeed, we observed the presence of tumor cells in intraocular tumor–associated lymphatics. However, note that there are limitations in detecting what may be a rare event by examining small sections of archival tissue at a single point in time. Although in this study the number of samples with tumor cell clusters in intraocular lymphatic vessels was small, the association with increased risk of lymph node metastasis and poor prognosis for survival was significant, reflecting the capability of intraocular melanoma cells to invade newly formed intraocular lymphatic vessels. Even if ciliary body melanomas with extraocular extension already have access to preexisting conjunctival lymphatics for lymph node metastasis,2 it might be possible that lymphatic spread can start even in the eye into intraocular tumor–associated lymphatic vessels. However, these data suggest, but do not prove, that intraocular lymphatics actually are functional or are, indeed, involved in transport of tumor cells to the regional lymph nodes. This concept of functional efficiency of intraocular tumor–associated lymphatic vessels is further supported by the observation that extraocular lymphatic invasion alone was not significantly associated with lower metastasis-free survival rates. Only tumor invasion into intraocular and extraocular lymphatics revealed significantly higher mortality rates, most likely due to increased tumor malignancy (large tumor size, epithelioid tumor cell type, high mitotic rate, and closed connective tissue loops).1-4

The main weakness of the present study is the small number of included patients, qualifying the conclusions from statistical testing. Therefore, these results should be verified in larger prospective series. Furthermore, the findings from the present study may suggest a potential for novel combined antinflammatory and antilymphangiogenic therapies, such as bevacizumab (Avastin; Roche, Welwyn Garden City, England),26,28 or specific antilymphangiogenic therapies, such as anti–VEGF receptor 3 antibodies28 or integrin α5 blocking peptides,27 in patients with uveal melanomas who develop extraocular extension to prevent lymphatic spread of the tumor to regional lymph nodes.

In conclusion, intraocular tumor–associated lymphatic vessels may be of functional significance in ciliary body melanomas with extraocular extension. They are mostly proliferating and can be the target of cancer cell invasion. This lymphatic invasion seems to be associated with an increased risk of lymphatic spread and mortality in these patients.

Submitted for Publication: December 2, 2009; accepted January 25, 2010.

Correspondence: Ludwig M. Heindl, MD, Department of Ophthalmology and Eye Hospital, Ophthalmic Pathology Laboratory, Friedrich-Alexander University Erlangen-Nürnberg, Schwabachanlage 6, 91054 Erlangen, Germany (ludwig.heindl@uk-erlangen.de).

Financial Disclosure: None reported.

Funding/Support: This study was supported by Priority Research Project SFB 643: B10 from the German Research Foundation; the Interdisciplinary Center for Clinical Research; the Section of Ophthalmic Pathology, German Ophthalmological Society; and ELAN Fonds of the University of Erlangen-Nürnberg.

Additional Contributions: Gottfried O. H. Naumann, MD, provided expert histopathologic advice, concept, and review of the manuscript; Harald L. J. Knorr, MD, provided outstanding clinical and surgical assistance; Carmen Rummelt, MTA, provided excellent technical support; and Werner Adler, PhD, provided statistical consulting.

REFERENCES


©2010 American Medical Association. All rights reserved.