Tumor-associated lymphangiogenesis in conjunctival malignant melanoma

Paul Zimmermann*, Tina Dietrich*1,2, Felix Bock1, Folkert K. Horn1, Carmen Hofmann-Rummelt1, Friedrich E. Kruse1, Claus Cursiefen1

*both authors contributed equally and should be regarded as first authors

1Department of Ophthalmology, University Erlangen-Nürnberg, Germany
2Department of Ophthalmology, University Medical Center Regensburg, Germany

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Correspondence: Claus Cursiefen, MD, Dept. of Ophthalmology, University of Erlangen-Nürnberg, Schwabachanlage 6, 91054 Erlangen, Germany; Tel.: 004999131 85-34141; Fax: 004999131 85-36401; Email: ccursiefen@yahoo.com

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Abstract

Background: To evaluate whether tumor-associated lymphangiogenesis, i.e. the formation of new lymphatic vessels (LVs) induced by a tumor, occurs in and around conjunctival malignant melanoma (MM).

Methods: Clinical files and conjunctival specimens of 20 patients with histologically diagnosed conjunctival MM were analyzed. Sections were stained with LYVE-1 and podoplanin antibodies as specific lymphatic endothelial markers and Ki67 as proliferation marker. The tumor area and the area covered by LV (LVA), the LV number (LVN), and the LV density (LVD) were measured within the tumor and in the peritumoral area in digital images of the specimen. The LV results were correlated with the histopathological characteristics, tumor location, recurrence rate, mitomycin C therapy and presence of metastases.

Results: LVs were detected in all specimens within the tumor and peritumorally. Significantly more Ki67+ proliferating lymphatic endothelial cells were detected in the tumor and in the peritumoral tissue up to 300 µm compared to the surrounding normal conjunctiva (>300 µm distance). There was a slightly positive correlation between the tumor size and the LVN and LVA in the 50 µm zone adjacent to the tumor. We did not find significant correlations between LVs and histopathological and clinical characteristics (location, shape, relapses, metastases), possibly due to small sample sizes. Non-limbal tumors with involvement of tarsus or fornix showed a tendency of higher LVD compared to limbal tumors.

Conclusion: Conjunctival MMs display tumor-associated LV within and around the tumor. The MM seems to induce lymphangiogenesis not only in the tumor, but also in its proximity.

Introduction

Malignant melanomas (MMs) of the conjunctiva are associated with significant morbidity and mortality due to high rates of recurrence and metastasis \(^1,2\). The dissemination of the tumor is linked to regional lymph nodes with subsequent distant metastasis \(^3\). Compared to cutaneous MM, conjunctival MM is rare. The annual age-adjusted incidence rates (per million) vary from 0.15 in Asians to 0.5 in non-Hispanic Whites \(^4,5\).

Up to date, there are only few features recognized as prognostic factors for conjunctival MM: Tumor location, expansion, relapse, multifocal location, involvement of the surgical margins and tumor depth are known prognostic factors for metastatic disease \(^6,7\). Histopathological characteristics seem not to be consistently associated with the clinical outcome \(^8\).

The primary treatment of conjunctival MM is surgical: Complete excision with tumor-cell free margins represents the therapy of choice, but can not be sufficiently performed in cases of diffuse growth. Topical mitomycin C as adjunct therapy has been established \(^8\); cryotherapy, laser ablation, radiation treatment, and chemotherapy in case of metastasis represent additional treatment options for conjunctival MM.

Conjunctival MMs are rich in blood vessels, which play a role in systemic hematogenous metastasis. However, the main route of metastasis of conjunctival MM is lymphogenic: Ultrasonic examination of the draining lymph nodes or even surgical removal of the sentinel lymph nodes has been recommended. Up to now, it
was not known, whether conjunctival MMs also display significant tumor-associated lymphangiogenesis, i.e. whether the tumor induces formation of new lymphatic vessels.

The extent of lymph node metastasis is supposed to be a major determinant for prognosis and staging of tumors\(^9\) and it has been shown that tumor-induced lymphangiogenesis is a strong risk factor for tumor metastasis in different human cancers\(^{10; 9; 11-13; 3; 14}\). The importance of tumor-induced lymphangiogenesis for lymphogenic metastasis in cutaneous MM has been shown recently\(^{10}\).

Purpose of this study was to analyze whether conjunctival MMs also display tumor-induced lymphangiogenesis, which may represent a possible new prognostic factor. We used specific lymphatic endothelial markers to analyze the presence of LVs in the tumor itself and in the adjacent tissue and correlated these data with the clinical outcome and histopathological characteristics of the tumors.

**Material and Methods**

**Patients and conjunctival sections**

Clinical files and histological sections of conjunctival MMs of 20 patients who were treated at the Department of Ophthalmology of the University Erlangen-Nürnberg, Germany, between 1987 and 2005 were retrospectively analyzed. The files were screened and the documented treatment and follow-up was taken into consideration. The clinical outcome of all patients was re-evaluated at the end of 2006 and again 2008 by interviewing the patients’ general practitioners for any new progress of the disease since the last visit, especially for systemic metastasis.

**Lymphatic vessel staining (LYVE-1 and podoplanin)**

For staining of LVs LYVE-1 served as specific marker for lymphatic vascular endothelium. The preparation of the histological sections of conjunctival MMs was performed as described previously\(^{15}\). Briefly, tissue was fixed in neutral buffered formalin, embedded in paraffin and cut in 4 µm sections. After deparaffinization and rehydration, sections were digested with proteinase K (Dako, Hamburg, Germany), incubated for 10 minutes with horseradish peroxidase (HRP). Sections of conjunctival MMs were incubated for 30 minutes with a rabbit polyclonal antibody against human LYVE-1 (1:100; Dako, Hamburg, Germany) and HRP-conjugated secondary antibody before development with 3-amino-9-ethylcarbazole (AEC\(^*\)) substrate (red reaction product) or 3,3´-diaminobenzidine (DAB; brown product). Sections were counterstained with Mayer haemalaun (Chroma, Münster, Germany). Positive controls were performed on corneoscleral ring specimens and negative controls with control IgG.

Since LYVE-1 is also expressed on tissue macrophages\(^{16; 17}\) only clearly identifiable vessels with an erythrocyte-free vessel lumen were counted as LV and specimens were double-stained with podoplanin as second lymphatic endothelial marker.

For podoplanin immunostaining polyclonal rabbit anti-human antibody against podoplanin (1:200, Dako, Hamburg, Germany) was used, followed by biotinylated goat anti-rabbit IgG for 30 minutes and detection by a streptavidin peroxidase complex (using DAB/AEC\(^*\) as the chromogen substrate). Positive controls were performed as described above.
**Ki67 staining**
Sections of paraffin embedded specimens were double-stained with LYVE-1 and monoclonal antibody against Ki67 (clone MIB-1, Dako, Hamburg, Germany) as a specific marker for proliferating cells. LVs with at least 5 endothelial cells with nuclear Ki67 positivity were considered to be Ki67 positive.

**Mitomycin C treatment**
The additional topical mitomycin C treatment of conjunctival MM by eye drops is standardized in our department as two 14 day cycles with mitomycin C 0.02 % eye drops five times a day with a 14-day break. Some patients were not treated with mitomycin C eye drops due to allergy or refusal. To analyze the potential anti-lymphangiogenic effect of mitomycin C therapy, tumor specimens of patients, who received mitomycin C treatment and had excisions later on during their clinical course (because of new suspect lesions) were compared to the specimens obtained before mitomycin C treatment.

**Microscopy and computer-assisted vessel analysis**
Histological sections of conjunctival MMs of 20 patients were taken into consideration. Sections were analyzed with a light microscope (BX51, Olympus Optical Co., Hamburg, Germany) and digital color images were taken with a 12-bit CCD camera (Color-View I, Olympus, Hamburg, Germany; 40x and 100x magnification). Analyses were performed using Cell^F (Olympus, Hamburg, Germany) and Image J analyzing program (available via http://rsb.info.nih.gov/ij/download.html). Morphometrical LV analysis was performed for the area of the tumor, the adjacent 50 µm zone, the mid-peripheral zone (50 -200µm), the peripheral zone (200-300µm) and the conjunctiva more than 300 µm away from the tumor border (defined as normal conjunctiva). If the tumor adjacent area was not completely represented on the specimen, we evaluated the area as far as represented. The tumor size was measured as the area covered by the tumor in the histological section. We determined the following parameters: 1. the LV number (LVN), 2. the area covered by LVs (LVA), 3. the LV density (LVD), determined by measuring the LVN and dividing it by the tumor cross sectional area (mm²).

**Functional and statistical analysis**
To determine statistical significance, quantitative analyses of the LVA , LVN, and LVD in all analyzed areas (intratumoral, 50 µm, 50 - 200 µm, 200 - 300 µm and >300 µm peritumoral) were performed in a standardized procedure using the statistic program InStat 3 (GraphPad Software Inc, San Diego, California, USA). Analyses were performed using the non-parametric test for the Ki67 analysis and the Pearson rank correlation for the correlation of tumor area to LVN and LVA.

**Results**

**Patients and histopathological characteristics**
The median age of the patients in the study was 70.4 years (43-100 years). 9 women and 11 men were treated. The MM of 13 patients was based on primary acquired melanosis (PAM); in 7 patients the origin of the MM remained unclear. The primary treatment was surgical, 10 patients had an additional topical mitomycin C treatment. The primary tumor was located in the fornix (2 patients), the tarsus and the upper lid (5 patients) or at the limbus/epibulbar conjunctiva (7 patients). 6 patients showed a
widely disseminated tumor, including some who have had primary excision outside of our department, so that the primary tumor location was not known. 10 patients showed a diffuse, 5 patients a nodular, and 5 a mixed growing type of the MM. The histopathological characteristics were: 13 tumors of mixed cell type, 5 tumors of spindle cell type, and 2 tumors of epitheloid cell type.

8 of the 20 patients showed more than 5 relapses during the clinical course. 5 patients suffered from metastasis: 1 patient was diagnosed for gastric metastasis 7 years after primary diagnosis, two patients for submandibular and neck spreading after 2 and 3 years, 1 patient for craniopharyngeal metastasis after 1 year and 1 patient had parotical metastasis after 14 years.

**Conjunctival MMs display intra- and peritumoral LVs**

Using LYVE-1 and podoplanin staining, we identified LVs in all included MM specimens, both within the tumor itself and in the adjacent tissue (**Figure 1**). There was a similar staining pattern for both lymphatic vascular endothelial markers in the conjunctival MM specimens.

**Conjunctival MMs are associated with intra- and peritumoral lymphangiogenesis**

To examine whether conjunctival MMs induce formation of new LVs, Ki67 staining was performed to detect proliferating lymphatic endothelial cells in the conjunctival MMs and the adjacent conjunctival tissue. Immunostaining with Ki67 revealed significantly more proliferating lymphatic endothelial cells in the tumor and in the directly adjacent conjunctiva compared to the peripheral zones. Non-parametric tests were performed for each zone separately with the following results concerning the ratio of Ki67-positive LVs: 1. tumor vs. tumor adjacent conjunctiva (50 µm zone) p=0.063 (not significant), 2. tumor vs. mid-peripheral zone (50 -200 µm) p=0.021, 3. tumor vs. peripheral zone (200-300 µm) p=0.031, 4. tumor vs. distant, presumably normal conjunctiva >300 µm from the tumor border p=0.002 (**Figure 2**). The results support the hypothesis of tumor-associated active lymphangiogenesis in the tumor and its proximity.

**Influence of tumor cross-sectional area on LVs**

We analyzed whether the extent of lymphangiogenesis in and around conjunctival MM was correlated with the tumor area. Therefore analyses of the LV parameters LVA, LVN, LVD were performed in the tumor and in the tumor environment (50 µm zone, 50-200 µm zone, 200-300 µm zone). Intratumoral LVN, LVA and LVD were not positively correlated to the tumor cross-sectional area (results for LVN and LVA shown as table in **Fig. 3**). The LVN and LVA in the 50 µm area directly adjacent to the tumor are positively correlated to the tumor cross sectional area; the results for the LVN demonstrated as scatter plot in **Fig. 3** (Pearson correlation coefficient r=0.64; p=0.002). There was a slightly inverse correlation for the LVD in the tumor with the tumor cross-sectional area (r=-0.257, p=0.237).

**Effect of mitomycin C therapy on LVs**

We analyzed the potential effect of topical mitomycin C treatment on LV formation. Specimens of patients (n=4) who underwent topical mitomycin C treatment after tumor excision and had subsequent excisions for tumor recurrence were analyzed for LVD and LVN (**Figure 4**). In 3 patients the specimens represented all 5 zones; in one patient the specimens showed only tumor without surrounding conjunctiva because of diffuse tumor growth (therefore only the tumor zone was analyzed in this patient).
Because of the limited number of patients, statistical analysis was restricted to descriptive analyses (Figure 4). Additional LV analysis (LVD, LVN, LVA) of specimens from mitomycin C treated patients compared with specimens from patients without mitomycin C treatment did not show statistically significant results.

Analysis of LVs in dependence of tumor location
Analyzing the location of the tumor, 7 patients had limbal/epibulbar tumors, 7 patients had a MM at the fornix or tarsus and 6 patients had a disseminated MM. The analyses of LV parameters (LVN, LVA, LVD) did not show statistical significant results in these small sample sizes. Because of the small number of patients, we performed only descriptive analyses as scatter plot. Fig. 5 demonstrates the LVD in dependence of the tumor location: In 4 of 7 patients with non-limbal MMs with involvement of the fornix or tarsus the LVD was higher than in all limbal tumor specimens (n=7).
Discussion

This study on conjunctival MM shows for the first time that conjunctival—and not only cutaneous—MMs display tumor-associated lymphangiogenesis.

In our study we found LVs in the tumor itself as well as in the peritumoral area. These erythrocyte-free LVs are stained with two new markers specific for lymphatic vascular endothelium, which are LYVE-1 and podoplanin. Most remarkably, the degree of actively proliferating, Ki67 positive lymphatic vascular endothelial cells is significantly higher within the MM and in the close vicinity of the tumor compared to normal, resting conjunctival LVs more distant from the tumor site. The ratio of Ki67 positive LVs is reduced with growing distance from the tumor border which supports the hypothesis of active tumor-induced formation of new LVs (tumor-associated lymphangiogenesis). It has been shown recently that lymphangiogenic growth factors, which are secreted by a primary tumor, can induce lymphangiogenesis²⁰-²³.

In our small pilot study there was a no significant correlation between tumor size (two-dimensional) and LVN and LVA in the tumor. The analyses of the tumor environment revealed a higher LVN and LVA in the 50 µm zone directly adjacent to the tumor, possibly being an indicator for active lymphangiogenesis in the tumor surroundings. We found a slightly inverse correlation between LVD in the tumor and the tumor cross sectional area. On the one hand that finding might be related to the two-dimensional calculations of the tumor area based on cross sectional specimens of the tumor. On the other hand small tumors might show a higher LVD as a signal for their starting potency of dissemination, possibly related to high secretion rates of lymphangiogenic growth factors. Another possible aspect is a centrally developing necrosis in larger tumors which might reduce the rate of lymphangiogenesis and cause reduced LVD. Furthermore, some studies describe a high interstitial pressure within tumors that promotes LV collapse¹³; thus compressed LV in larger tumors might appear smaller than LV in smaller tumors.

We analyzed the putative impact of lymphangiogenesis on recurrence rate and metastasis, but did not find a significant correlation, possibly related to the small sample sizes. Larger (prospective) studies now will have to evaluate tumor-associated lymphangiogenesis as a putative risk factor for tumor metastasis. Tumor location is one of the clinically most important prognostic predictors of conjunctival MM: non-limbal tumors show a higher incidence of initial systemic metastasis and reduced survival rates⁵;⁷. This fact might be due to facilitated access to blood vessels or to the draining LVs. Our descriptive analysis of the tumor location, i.e. limbal vs. palpebral/fornix vs. disseminated conjunctival in correlation with parameters of lymphangiogenesis showed a tendency of higher LVD in non-limbal palpebral/fornix tumors. We did not find significant differences concerning the lymphangiogenic parameters in correlation with the different growth patterns i.e. shape of conjunctival MM. Additional studies are necessary to elucidate these aspects.

Topical mitomycin C treatment might provide an antilymphangiogenic effect as it has been suggested in several other studies on the treatment of conjunctival MM¹⁸;⁸. We performed only descriptive analyses due to the small sample sizes of histological tumor specimens after mitomycin C treatment: There was a tendency of reduced LVD in specimens from patients after mitomycin C therapy compared to specimens before mitomycin C therapy, while LVN and LVA were not reduced. Nevertheless, other factors as fibrotic tissue-remodeling after surgical excision, the influence of co-
medications as topical steroids or other causes can not be ruled out and may be of significant influence.

The prognostic importance of intra- and peritumoral lymphangiogenesis is becoming more established by a growing number of studies on several human cancers. The identification of high risk patients is helpful in order to individually optimize screening and treatment guidelines. The extent of tumor-associated lymphangiogenesis in conjunctival MM may be one novel prognostic criterion besides other known or putative risk factors. Immunostaining for lymphatic markers as LYVE-1 and podoplanin in routine histological work-up of tumor samples might be warranted if future studies reveal a correlation between tumor-induced lymphangiogenesis and prognosis of the tumor in terms of metastasis and recurrence rate. The histological analysis of lymphangiogenesis parameters compared with sentinel lymph node biopsy as a mean of guiding treatment and follow-up is under discussion.

This study on tumor-associated lymphangiogenesis may pave the road to new targets for innovative therapeutic approaches and serve as a novel prognostic parameter in conjunctival MM. Much effort in anti-lymphangiogenic and anti-angiogenic research has been made to develop new therapeutic approaches to inhibit tumor spreading. Recently, different assays of selective inhibition of LV growth on the ocular surface have been shown. In the future anti-lymphangiogenic treatment options might help to minimize the risk of metastasis in conjunctival MM.

**Conclusion**

MMs of the conjunctiva display LVs within and around the tumor. There is evidence for tumor-induced lymphangiogenesis, i.e. formation of newly formed LVs. These LVs may act as conduits for tumor metastasis. Lymphangiogenesis parameters as LVD and LVN may become useful novel prognostic indicators for conjunctival MM. Novel anti-lymphangiogenic therapeutic strategies may contribute to optimize the therapy of conjunctival MM in the future.

**Figure legends**

**Figure 1: Tumor-associated lymphatic vessels in MMs of the conjunctiva**

**a)** Representative image of LV staining with LYVE-1 antibody as specific marker for lymphatic endothelium: LYVE-1 positive peritumoral LVs. **b)** Representative image of podoplanin stained LVs in the tumor adjacent conjunctiva. **c)** Intratumoral LYVE-1 positive LVs (magnification x100/x200). Area of primary acquired melanosis is marked with an asterisk.

Arrows: The LYVE-1/podoplanin stained lymphatic vessels (arrowhead) Note that erythrocyte filled blood vessels are not stained with these lymphatic endothelial specific markers.

**Figure 2: Tumor-induced lymphangiogenesis: Significantly more proliferating LVs were found intratumorally and next to the tumor than in distant conjunctiva (> 300 µm).**

Representative images of conjunctival MM specimen stained with LYVE-1 and proliferation marker Ki67. Ki67 positive cells are marked (arrowhead). **a)** Representative image of Ki67 positivity in tumor-associated lymphatic endothelial
cells; red: Ki-67; brown: LYVE-1 (magnification x1000) b) Significantly more proliferating LVs were found intratumorally as well as in the directly adjacent tumor environment than in the more distant conjunctiva. Paired t test was performed for each zone separately with the following results: 1. tumor vs. tumor adjacent conjunctiva (50 µm zone) p=0.063 (not significant), 2. tumor vs. mid-peripheral zone (50-200 µm) p=0.021, 3. tumor vs. peripheral zone (200-300 µm) p=0.031, 4. tumor vs. conjunctiva >300 µm from the tumor border p=0.002; n=20, median is marked; circles mark 3 outliers. The results support the hypothesis of tumor-associated active lymphangiogenesis in the proximity of the tumor.

Figure 3: Influence of tumor cross-sectional area on LVs
Analyses of the LV parameters LVA, LVN, LVD were performed in the tumor and in the tumor environment (50 µm zone, 50-200 µm zone, 200-300 µm zone). Intratumoral LVN, LVA and LVD were not positively correlated to the tumor cross-sectional area (a). There was a slightly inverse correlation for the LVD in the tumor with the tumor cross-sectional area (r=-0.257, p=0.237). The LVN in the 50 µm area directly adjacent to the tumor was positively correlated to the tumor cross sectional area (Pearson correlation coefficient r=0.64; p=0.002), demonstrated as scatter plot (b).

Figure 4: Effect of topical mitomycin C on LVs
Scatter plot diagram of the measured LVD in the tumor (0), in the adjacent 50 µm zone (50), in the 50-200 µm zone (200), in the 200-300 µm zone (300) before and after mitomycin C therapy. LVD before mitomycin C therapy is marked as ring, LVD after mitomycin C therapy is marked as asterisk. Sections of 4 patients were analyzed, in one patient there was no surrounding conjunctiva represented on the specimen and only the tumor area was analyzed. Because of the limited number of patients descriptive analyses were performed.

Figure 5: Analysis of LVs in dependence of tumor location
Descriptive scatter plot: In 4 of 7 patients with non-limbal MMs with involvement of the fornix or tarsus the LVD was higher than in all limbal/epibulbar tumor specimens (n=7). 7 patients had limbal/epibulbar MMs, 7 patients had a MM at the fornix or tarsus and 6 patients had a disseminated MM. The analyses of LV parameters (LVN, LVA, LVD) did not show statistical significant results in these small sample sizes.

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Competing interest: None declared.

References
Figure 1
Figure 2

b) Proliferative Lymphatic Vessels

- Intratumoral
- 50μm peritumoral
- 50-200μm peritumoral
- 200-300μm peritumoral
- >300μm peritumoral

Ratio of KI 67+ vessels (%)

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n=20
Figure 3

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<tr>
<td>200-300 μm peritumoral area</td>
<td>p=0.047; r=0.438</td>
<td>p=0.066; r=0.499</td>
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b)
Figure 4

LVD (Vessels/mm²) vs. Area of analyzed LVD (μm)

- LVD before MMC
- LVD after MMC
Figure 5

![Graph showing LVD (vessels/mm²) across different regions]

Limbal/Epibulbar  Fornix/Tarsus  Disseminated

LVD (vessels/mm²)

0.00, 5.00, 10.00, 15.00, 20.00, 25.00