Lack of Lymphangiogenesis Despite Coexpression of VEGF-C and Its Receptor Flt-4 in Uveal Melanoma

Ruud Clarijs, Lia Schalkwijk, Dirk J. Ruiter, and Robert M. W. de Waal

PURPOSE. Because lymphatic vessels are absent from the normal eye and because uveal melanomas are presumed to spread by a hematogenous route in the absence of tumor exposure to conjunctival lymphatics, this study was undertaken to investigate the presence of lymphatic vessels in primary uveal melanomas.

METHODS. The presence of lymphatics in 2 control eyes and in 33 primary uveal, 10 primary cutaneous, and 3 metastatic cutaneous melanomas was evaluated by using a double-immunostaining protocol that differentially highlights blood and lymphatic vasculature. In addition, 14 uveal melanomas were immunostained for the lymphatic growth factor vascular endothelial growth factor (VEGF)-C (with anti-VEGF-C polyclonal antibodies [pAbs]), its receptors Flt-4 (with monoclonal antibody [mAb] 9D9) and KDR (with anti-KDR mAb [Clone KDR-2]), and the hemangiogenic factor VEGF-A (with anti-VEGF pAbs).

RESULTS. Lymphatics were not detected in normal eyes or in uveal melanomas. As a consequence, signs of lymphangiogenesis were not present. There was coexpression of VEGF-C with Flt-4 and KDR in 6 (43%) of the 14 melanomas. Staining for VEGF-A was completely negative in 25 uveal melanomas analyzed.

CONCLUSIONS. The strictly hematogenous metastasis of primary uveal melanomas is explained by the absence of lymphatics in and around the tumor. The current data suggest that, in the presence of endothelial Flt-4, VEGF-C expression is not sufficient to induce lymphangiogenesis from preexisting blood vessels in human cancer. (Invest Ophthalmol Vis Sci. 2001;42:1422–1428)

Primary uveal melanoma is the most common malignant intraocular tumor. It has a marked metastatic preference for the liver. Once metastasis occurs, prognosis becomes very poor. Microvessel density was identified as an important prognostic factor for many types of tumors. However, in the case of uveal and cutaneous melanomas, there are conflicting reports on the presence of an association between microvascular density and prognosis. In uveal melanoma, blood vessel architecture, and both architecture and density, have been related to prognosis.

Dissemination of primary intraocular and posterior uveal melanoma occurs exclusively by a hematogenous route. Anterior uveal melanomas may also metastasize to local cervical lymph nodes after invading the conjunctival lymphatics. The restricted hematogenous metastasis is generally explained by the intraocular absence of lymphatics and the extrapolated assumption that, this vessel type is therefore absent in uveal melanoma as well. However, this hypothesis has never been confirmed. In skin, lymphatic vessels are abundant, which explains the phenomenon of locoregional metastasis that is frequently observed in cutaneous melanoma. It is therefore possible that the mere presence of pre-existent lymphatics in skin contributes to the difference in metastatic pattern between uveal and cutaneous melanoma. Furthermore, the presence of lymphatics in cutaneous melanoma may obscure the relation between blood vessel density and prognosis that was established in uveal melanoma. However, induction of lymphatic vessel formation (lymphangiogenesis) may play a role. Angiogenesis is necessary for tumor growth. In this process, the role of vascular endothelial growth factor (VEGF)-A has been firmly established. Another VEGF family member, VEGF-C, has been identified as a lymphatic endothelial growth factor. VEGF-C expression has been observed in tumor cells, which theoretically opens the possibility that tumors, besides hemangiogenesis, also induce lymphangiogenesis. During embryogenesis, lymphatics arise from venous endothelial cells, but whether veins can be the source of lymphatic neovascularization during adult life, should this occur, is unknown. Because VEGF-C exerts part of its function through the tyrosine kinase receptor Flt-4, and because this receptor is upregulated in blood vessels of certain tumor types, analysis of Flt-4 expression in uveal melanoma may be relevant as well. Should lymphatics in uveal melanomas be absent, VEGF-C expression in this type of tumor might influence hemangiogenesis, as reported in animal studies.

To elucidate the nature of the vasculature in uveal melanoma and its role in mediating growth and metastasis, knowledge of the presence of a lymphatic vasculature becomes very relevant. In the present study, we evaluated the presence of lymphatics in primary uveal melanoma and in primary and cutaneous metastatic lesions of cutaneous melanoma by using a double-immunostaining protocol (using the blood vessel endothelial marker PAL-E and the panendothelial marker CD31) that differentially highlights blood and lymphatic vasculature. In addition, we studied the expression of VEGF-A, VEGF-C, and the receptors KDR and Flt-4, the latter of which is believed to be specific for VEGF-C.

MATERIALS AND METHODS

Specimens
Frozen specimens of 33 primary uveal melanomas, 10 primary cutaneous melanomas, 3 cutaneous metastatic lesions of cutaneous melanoma, 2 uninvolved eyes, 1 invasive ductal breast carcinoma, 1 hemangioendothelioma, and 1 normal preputial skin (Table 1) were obtained from the pathology archives of the University Hospital (Nijmegen) where they had been stored at −130°C. Presence or absence of disease in all specimens had been determined by a pathologist. All primary melanomas and the uninvolved eyes were obtained by surgery. The uveal melanomas varied from 5 to 28 mm in diameter (median, 17 mm) and included 31 choroidal and 2 ciliary melanoma lesions. Uveal melanoma lesions were divided in two parts along the maximal diameter. One part was formalin fixed and the other part was snap frozen. By using hematoxylin and eosin staining on paraffin sections, the uveal melano-
mas were classified as 11 spindle cell type and 22 epithelioid and mixed type. Azan staining without counterstaining on unbleached paraffin sections showed that 15 uveal melanomas contained the arc, loop, and network matrix patterns (Fig. 1: PAS-positive patterns, as described recently8,23).

Antibodies
mAbs used for immunohistochemistry (Table 1) included anti-CD34 (QBEnd/10; Dako, Glostrup, Denmark), the blood vessel endothelial marker mAb PAL-E (undiluted supernatant, our laboratory), the panendothelial marker CD31 (PECAM-1; British Biotechnology, IKT Diagnostics, Uithoorn, The Netherlands), anti-α-smooth muscle actin (α-SM1; Sigma Chemical Co., St. Louis, MO), anti-KDR (Clone KDR-2; Sigma Chemical Co.), and anti-Flt-4 (9D9; Molecular/Cancer Biology Laboratory, University of Helsinki, Finland). For VEGF-A and VEGF-C stainings, polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used.

Immunochemistry
Four-micrometer cryosections were air dried and fixed in acetone at room temperature for 10 minutes. After incubation steps, sections were rinsed with ample phosphate-buffered saline (PBS). For the primary melanomas, each analysis included six successive sections of each specimen incubated with QBEnd/10 (diluted: 1:100), PAL-E (undiluted supernatant), and anti-KDR (diluted 1:400), 9D9 (diluted: 1:1000), and the pAbs to VEGF-A (diluted 1:20) and VEGF-C (diluted: 1:20) for 60 minutes at room temperature. Then, secondary 1:200 diluted biotinylated affinity-purified anti-mouse IgG (for CD34, PAL-E, KDR, and Flt-4; Vectastain; Vector Laboratories, Burlingame, CA) or affinity-purified anti-rabbit IgG (for VEGF-A and VEGF-C) was incubated for 30 minutes, followed by a 45-minute incubation (for CD34, PAL-E, and VEGF-A) or 30-minute incubation (for Flt-4, KDR, and VEGF-C) with peroxidase-labeled biotin-avidin complex (Vectastain; Vector Laboratories). Subsequently, the KDR, Flt-4, and VEGF-C sections were incubated for 10 minutes with biotinylated tyramine (dilution 1:200), followed by a 20-minute incubation with ABC-peroxidase solution (catalyzed reporter deposition method21). All stainings were developed by a 10-minute incubation with 0.4 mg/ml levamisole (Sigma-Aldrich, Bornem, Belgium). In control sections, primary antibodies were omitted. Positive controls for all antibodies were included (Table 1). The QBEnd/10, KDR, VEGFA, VEGF-C, and 9D9 stainings were counterstained for 45 seconds with Harris’ hematoxylin (Merck, Darmstadt, Germany) at room temperature. All sections were mounted in medium (Immsolt; Klinipath BV, Duiven, The Netherlands).

To validate our PAL-E/CD31 double-staining protocol, we additionally stained serial sections of preputial skin by anti-CD34 mAb and by mouse anti-human α-smooth muscle actin mAb (diluted 1:15,000). Masson trichrome histochemistry was performed as well, on an adjacent section.

By including positive controls, stainings of the normal and tumor tissues were validated. To exclude exogenous peroxidase activity or nonspecific background, all serial stainings were incubated in a mixture of 1 ml 30% H2O2 in 200 ml acetone during fixation for 5 minutes and blocked by incubation with 20% normal horse serum (for PAL-E, CD34, KDR, Flt-4, α-SM1) or 20% normal goat serum (for VEGF-A and VEGF-C).

RESULTS
Vascular Staining in Human Skin
To validate vascular staining protocols, preputial skin was used, because of its richness in lymphatics. As described previously,21,22 the PAL-E/CD31 double-staining design was based on the reactivity of anti-CD31 mAb with both lymphatic and blood vessel endothelial cells, in combination with the reactivity of PAL-E with blood vessel endothelium alone. The blood vessel endothelial staining produced initially by anti-CD31 is overruled by staining by PAL-E. Thus, the vasculature was differentially highlighted in preputial skin sections (Fig. 2A). We found strong PAL-E positivity of blood capillaries and venules, but no staining of arterial vessels. The CD31 antibody staining that was not masked by PAL-E, stained both fine, thin-walled capillaries and venules. PAL-E- and CD31-positive (PAL-E+ve) vessels were classified as blood vessels and PAL-E-negative and CD31-positive (CD31+ /PAL-E−) ones were classified as lymphatic (or arterial) vessels.22,25,26 The blood vessel specificity of the differential staining was confirmed by the presence of smooth muscle cells (Figs. 2A, 2B). Masson trichrome histochemistry, highlighting elastic fibers in blood vessel walls, confirmed this specificity (Fig. 2D). Because arteries and lym-

**TABLE 1. Antibodies and Positive Controls Used for Immunohistochemistry**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>Type of Endothelium</th>
<th>Tissue</th>
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<tbody>
<tr>
<td>CD34</td>
<td>QBEnd/10</td>
<td>Blood vessel</td>
<td>Preputial skin, cutaneous melanoma (median, submitted for publication)</td>
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<tr>
<td>α-SM1</td>
<td>Vascular</td>
<td>Blood and lymphatic vessel</td>
<td>Preputial skin</td>
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<tr>
<td>CD31</td>
<td>PECAM-1</td>
<td>Venous blood vessel and capillaries, not cerebral and retinal vessels*</td>
<td>Preputial skin, cutaneous melanoma</td>
</tr>
<tr>
<td>Flt-4</td>
<td>Clone KDR-2</td>
<td>Blood vessel</td>
<td>Hemangioma</td>
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<td>VEGF-A</td>
<td>VEGF-A Abs</td>
<td>Lymphatic in normal tissues</td>
<td>Preputial skin, cutaneous melanoma</td>
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<tr>
<td>VEGF-C</td>
<td>VEGF-C Abs</td>
<td>Lymphatic and blood in tumor tissues</td>
<td>Preputial skin, cutaneous melanoma</td>
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* Venous vessels that form a blood–brain barrier29 or blood–retinal barrier.27
phatics are both negative for PAL-E, it is not always possible to differentiate these vessel types. As we have recently demonstrated (Clarijs et al., submitted for publication), an anti-CD34 mAb stained the PAL-E blood vasculature and CD31/PAL-E arteries, whereas the CD31/PAL-E lymphatic vessels were negative for CD34 (Figs. 2A, 2C). Using this combination of stains, all vessels could be classified.

Vascular Staining in Normal Eyes

Frozen specimens of the ocular wall of two normal eyes were evaluated. In the choroid layer, PAL-E blood capillaries and venules were observed (Fig. 3A). As demonstrated previously, no PAL-E positivity of the vasculature in the retinal layer containing the blood-retinal barrier was observed. Because all CD31/PAL-E vessels were stained by the anti-CD34 mAb (Fig. 3B), these were identified as arterial vessels. No CD31/PAL-E lymphatics were observed in the choroid layer.

Vascular Staining in Cutaneous and Uveal Melanomas

In all primary uveal (n = 33) and cutaneous melanomas (n = 10) and in cutaneous metastatic lesions of cutaneous melanomas (n = 3), PAL-E blood capillaries and venules were observed, whereas CD31/PAL-E arteries were present in only three uveal melanoma lesions. In the primary and metastatic cutaneous melanomas, CD31/PAL-E lymphatics were observed in the pre-existent skin directly surrounding the tumor (Fig. 4A) and delicate lymphatics between tumor fields, as described in more detail previously. In uveal melanomas, all PAL-E blood capillaries and venules and CD31/PAL-E arteries were also stained by the anti-CD34 mAb. In none of the uveal melanomas, were CD31/PAL-E lymphatics observed. A representative example is shown in Figures 4B and 4C. Vascular staining by the PAL-E mAb was superior to staining by the anti-CD34 mAb (Figs. 4C, 4D). No evident staining of uveal melanoma cells by any of the endothelial markers was observed.

**Figure 1.** Evaluation of matrix patterns in a paraffin-embedded primary uveal melanoma specimen stained by azan histochemistry. Extracellular matrix and nuclei of tumor cells were highlighted. Loops and network patterns were clearly visualized. (B) Higher magnification demonstrating the clear decoration of the matrix patterns. Magnification, (A) ×100; (B) ×400.

**Figure 2.** Immunohistochemical analysis of the vasculature in a preputial skin section shows differential staining of blood and lymph vessels. Blood vessel capillaries and venules are stained red by the PAL-E mAb, lymph vessels and arteries blue by the anti-CD31 mAb (A). (¢) Corresponding lymphatics in (A), (B), and (C); arrows: two arteries; arrowhead: vein. (B) Staining by an anti-α-smooth muscle actin mAb demonstrated the absence of smooth muscle cells in lymphatics, whereas all blood vessels were positive. (G) Anti-CD34 staining was selectively confined to the blood vasculature including arteries (arrows). Numerous stromal cells were CD34−, as well. (D) Masson trichrome histochemistry of the boxed area (A) is depicted, showing evident differences in vessel morphology (in a modified protocol, connective tissue stained green instead of blue). In the lymphatic vessel wall (¢) only endothelial cells were present. (B, C) Counterstained with Harris’ hematoxylin. Magnification, (A–C) ×100; (D) ×250.
Staining for VEGF-A was completely negative in series of 25 uveal melanomas. In total, 14 uveal melanomas were evaluated for VEGF-C, KDR, and Flt-4 expression. Table 2 summarizes all staining results. Eight tumors were positive for expression of VEGF-C (Fig. 5A), and six tumors were negative. In the positive tumors, distinct areas of positive cytoplasmic staining for VEGF-C were observed in the tumor cells directly surrounding Flt-4 blood vessels (Figs. 5A, 5B). VEGF-C expression colocalized with blood vessel endothelial Flt-4 expression in seven tumors and in six of those, with expression of endothelial Flt-4 and KDR (Fig. 5), whereas in one VEGF-C-positive tumor, neither Flt-4 nor KDR expression could be detected (melanoma 2, Table 2). In nine tumors, endothelial Flt-4 expression was observed, and in two of those, no evident VEGF-C staining was detected (melanomas 1 and 4, Table 2). In one tumor sample, two separate nodules were present, in one of which VEGF-C, KDR, and Flt-4 expression was observed, whereas in the other nodule these stainings were negative (melanoma 13, Table 2).

**TABLE 2. Overview of the Expression of VEGF-C, Flt-4, and KDR and the Presence Loops and Network Patterns in 14 Uveal Melanomas**

<table>
<thead>
<tr>
<th>Melanoma</th>
<th>VEGF-C</th>
<th>Flt-4</th>
<th>KDR</th>
<th>Loops and Networks</th>
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<tr>
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<td>Mean</td>
<td>57%</td>
<td>64%</td>
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<td>95% CI</td>
<td>28–83%</td>
<td>35–88%</td>
<td>23–77%</td>
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DISCUSSION

To address the question of whether the absence of lymphatic spread in primary uveal melanoma can be explained by the absence of lymphatic vasculature, we evaluated the presence of lymphatics and the expression of VEGF-C and Flt-4 in this type of tumor. Our staining approach made it possible to differentiate between lymphatic and blood vasculature, which confirmed our recent analysis in a variety of normal tissues and carcinoma types and was validated in the present study by additional α-smooth muscle immunostaining and Masson stains.
trichrome histochemistry. However, it is known that PAL-E is present on capillary and venous endothelium throughout the body, with the exception of vessels in areas in the brain with an intact blood–brain barrier. In the eye, there is a similar barrier (i.e., blood-retinal), and endothelial PAL-E antigen expression is absent there as well. In areas in both the brain and the eye where such a barrier is not present, the PAL-E antigen is expressed on the endothelium. Therefore, it was not possible to evaluate blood and lymphatic staining by the PAL-E/CD31 double-staining protocol in normal eyes alone. Because anti-CD34 mAb detects all types of vessel endothelium with the exception of the lymphatic vasculature (Clarj et al., submitted for publication and Ref. 31), blue (CD31+/PAL-E-) vessels could be classified as blood or lymphatic vessels by comparison with CD34 expression. In uveal melanomas, all vessels were stained by PAL-E or CD34, confirming earlier results. Thus, in this way, we were able to classify both types of vessels in preputial skin, normal eye, and uveal melanoma sections.

In our specimens, the extent of melanin pigmentation did not interfere with the detection of the vascular patterns, making bleaching unnecessary. Azan histochemistry resulted in deep blue staining of extracellular matrix and red staining of the cell nuclei, whereas PAS histochemistry is less powerful in extracellular matrix component detection. Indeed, for this approach, bleaching of melanoma sections and the use of a green filter during microscopy are required for reliable evaluation. However, in case of the presence of strongly pigmented melanophages along the matrix patterns (as seen in Fig. 4), bleaching may still be necessary to differentiate between melanophages and the azan-positive blue matrix patterns. The use of azan histochemistry in identifying matrix patterns was confirmed by immunofluorescence and electron microscopy (data not shown).

Electron microscopy has suggested that endothelium-free channels are lined by basement membrane. Although PAL-E detects the epidermal basal membrane, this is a specific pattern and staining of basal membranes of endothelium-free channels in the fibrovascular patterns is not likely to occur. However, at low magnification, the PAL-E/CD31 double staining may have given the impression that in addition to blood vessels, certain parts of fibrovascular patterns were identified. Evaluation at higher magnifications (Figs. 4C, 4D) demonstrated, however, that this impression was caused by the presence of numerous melanin-laden macrophages spread along the vasculature. Furthermore, vascular detection by PAL-E mAb was superior to anti-CD34 mAb in all uveal melanomas and in those melanoma lesions that did not contain arc, loop, and network patterns. In conclusion, the PAL-E/CD31 double-staining protocol could not be used to evaluate the existence of endothelium-free channels.

Knowledge of the absence of lymphatics contributes to the evaluation of the existence and nature of the nonendothelialized blood-conducting channels. Although the existence of these channels is still controversial in uveal melanoma, the absence of lymphatics rules out the possibility that these channels were, in fact, lymphatic channels.

In the present study, lymphatics were absent from both the normal eye and the uveal melanoma specimens. This is in line with the absence of lymphogenous metastasis in intraocular and posterior uveal melanomas, leaving only the hematogenous route open for dissemination. Although prognosis is related to microvascular density in uveal melanoma, this correlation is absent in cutaneous melanoma. In addition to

**FIGURE 5.** Immunohistochemical analysis of VEGF-C (A), Flt-4 (B), and KDR (C) expression in uveal melanoma. VEGF-C expression is located in part of the tumor cells surrounding the Flt-4+ and KDR+ blood vasculature. Counterstained with Harris' hematoxylin. Magnification, ×200.

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blood vessels, lymphatics also are involved in indirect metastasis to the blood stream. Thus, the absence of lymphatics in uveal melanoma tumors and the presence of lymphatics in cutaneous melanoma makes it plausible that lymphatics also play a role in determining the rate of distant metastasis and prognosis. In this respect, it is an interesting question whether lymphatic vessel density, or rather a combination with blood vessel density, is related to prognosis and metastatic spread in cutaneous melanomas. However, because we were not able to evaluate microvascular density in relation to prognosis in our series, we cannot confirm this hypothesis.

Although blood vessel angiogenesis is an established phenomenon, it is unknown whether lymphangiogenesis occurs in human cancer. Because we cannot be absolutely sure that immature lymphatic vessels express lymphatic markers such as CD31 or Flt-4, we cannot rule out the occurrence of lymphangiogenesis in uveal melanoma. However, it is likely that the life-span of larger tumors would allow maturation of lymphatic vessels after lymphangiogenesis. These mature vessels would readily be detected by our staining protocol. Furthermore, Flt-4 expression is present on sprouting lymphatic vessels during wound healing in the adult, whereas lymphatic vessels remain PAL-E during development. In our series, we did not observe such Flt-4 and PAL-E vessels. Thus, our study strongly suggests that lymphangiogenesis does not occur in this type of tumor, although we cannot rule it out completely.

VEGF-C has been identified as a lymphatic endothelial growth factor during embryogenesis. In the present study, although VEGF-C was expressed and Flt-4 blood vessels were present concurrently as a source of endothelial cells (for review see Ref. 38) lymphangiogenesis did not occur in uveal melanomas. VEGF-C is, however, also able to induce hemangiogenesis. In addition, its receptors KDR and Flt-4 are involved in angiogenesis (for extensive review, see Ref. 39). Therefore, the clear relation between the expression of VEGF-C, KDR, and Flt-4 suggests that the presence of VEGF-C and its receptors in uveal melanoma may contribute to hemangiogenesis. KDR is involved early in angiogenesis during embryogenesis, and its expression becomes upregulated on tumor endothelium under hypoxia (for extensive review, see Ref. 39) These data suggest that in tumor areas with local KDR expression, endothelium is in a state of angiogenesis, whereas in areas without KDR, no new vessels can be formed. Therefore, the coexpression of KDR and VEGF-C supports the role of VEGF-C as a hemangiogenic growth factor.

Many different types of tumors express VEGF-A, an important regulator of angiogenesis, indicating that tumor cells in addition to endothelial cells contribute to the tumor blood vasculature. Therefore, it was surprising that VEGF-A was absent from all uveal melanoma lesions, as demonstrated previously. Because of the absence of VEGF-A, it appears that hemangiogenesis in uveal melanoma may instead be driven by VEGF-C. In this respect, the presence of VEGF-B and VEGF-D should be further evaluated.

In conclusion, although the lymphatic endothelial growth factor VEGF-C and its receptor Flt-4 are expressed, neither lymphatics nor signs of lymphangiogenesis were present in normal eye and primary uveal melanomas, indicating that the concerted action of these players is not sufficient for lymphangiogenesis to occur in the adult in this type of tumor. Furthermore, hemangiogenesis in uveal melanoma is not associated with expression of VEGF-A, but may be driven by other angiogenic factors such as VEGF-C.

VEGF-C and Flt-4 Expression in Uveal Melanoma

Acknowledgments

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References


