Uveal Melanoma Cell Staining for CD34 and Assessment of Tumor Vascularity

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Purpose. Aggressive melanoma cells may express endothelial markers that can be used to calculate microvascular density (MVD). High MVD has been associated with adverse outcome in uveal melanoma. If tumor cells label with endothelial cell markers, then MVD may not accurately reflect a tumor's vascularity. This study was designed to study the influence of melanoma cell labeling by endothelial cell markers on MVD.

Methods. Tissue sections of 200 ciliary body or choroidal melanomas were stained with CD34 alone, and the MVD was calculated by counting discrete foci of CD34 labeling in hot spots, as described previously. From adjacent sections double labeled by fluorescent immunohistochemical stains for S100 protein and CD34, tumor cells labeling with both markers were identified. The relationship between marker coexpression and MVD was tested. Tissue sections were also double labeled for Melan-A and CD34.

Results. MVD was found to be associated with death from metastatic melanoma as reported previously. However, colocalization of both Melan-A and S100 protein with CD34 was demonstrated. The labeling of tumor cells by CD34 was associated with an elevated calculation of MVD ($P < 0.0001$) but not with cell type, mitotic figures, tumor-infiltrating lymphocytes, or PAS-positive patterns.

Conclusions. CD34 may label uveal melanoma cells and may contribute to computation of MVD. Although MVD is prognostically significant in uveal melanoma, this feature is not an exclusive measure of tumor vascularity. (*Invest Ophthalmol Vis Sci.* 2002;43:2533–2539)

Attention has been focused on angiogenesis as a critical pathway by which tumors acquire their blood supply. Recognizing that angiogenic responses may be focal and not evenly distributed throughout the tumor, investigators have gathered considerable evidence supporting an association between outcome and the number of blood vessels in hot spots identified in tissue sections stained for endothelial cell markers: microvascular density (MVD).[1] Although MVD is associated with outcome in many studies, other investigators have failed to demonstrate this association.[2] There is now evidence that markers for endothelial cells conventionally used in studies of tumor MVD may be expressed on tumor cells.[3–6] MVD is typically determined by counting discrete foci of labeling in tissue sections by putative endothelial cell markers.[7] Therefore, if tumor cells express these markers, then the attribute known as MVD may not be a pure quantitative measure of vascularity.

In uveal melanoma, high MVD has been associated with death caused by metastatic melanoma, by use of factor VIII–related antigen (FVIII-RAg)[8] and CD34[9] as markers for endothelial cells. However, it is of interest that the endothelial cell marker CD31 has been shown to label the nuclear matrix of uveal melanoma cells,[2] and CD34 labeling of melanocytes has been demonstrated recently in tissue sections of cellular blue nevi[6] and desmoplastic cutaneous melanoma.[4]

We therefore studied tissue sections of eyes removed for ciliary body or choroidal melanoma to further investigate the possibility of melanoma cell labeling by a putative endothelial cell marker and to examine the influence that expression of an endothelial cell marker on a tumor cell may have on the determination of MVD.

Materials and Methods

Immunohistochemistry: Exploratory Studies

To explore the possibility that melanoma cells labeled with CD34, tissue sections of test sample of 12 melanomas drawn from the archives of either the University of Illinois at Chicago or the University of Iowa that were formalin fixed were stained for CD34QBend10 (titer 1:14; Dako, Carpinteria, CA) and developed with the diaminobenzidine (DAB) chromogen followed by peroxide bleaching to remove melanin.[10] To eliminate the possibility that residual melanin after incomplete bleaching may be interpreted as positive labeling by CD34, sections adjacent to the ones labeled with CD34 were stained by the PAS-without-hematoxylin method after bleaching and compared.

To confirm the observation that melanoma cells in tissue sections may label for CD34, adjacent tissue sections to the test sample of 12 human uveal melanomas were stained for the presence of both S100 protein (titer 1:100, Dako), detected by labeling with a chromogen (Alexofluor 594; Molecular Probes, Eugene, OR) and CD34QBend10 (titer 1:14, Dako) labeled with a second chromogen (Alexofluor 488; Molecular Probes). All immunohistochemical reactions were performed in an automated immunohistochemical staining device (Dako) with appropriate negative and positive controls for each label; normal intraocular blood vessels adjacent to the tumor served as the internal control. To confirm CD34 labeling of melanoma cells, Melan-A was substituted for S100 protein in double-labeling immunofluorescence studies, as just described.

Immunohistochemical double-labeled preparations were examined with a microscope equipped for fluorescence (BX40; Olympus America, Melville, NY). Digital images were captured (Optronics MagnaFire Camera; Optronics, Goletta, CA) separately for each chromogen, and the images were merged with the software accompanying the camera (MagnaFire, ver. 2; Optronics).

Assessment of MVD

We used CD34 to determine MVD in tissue sections from a previously reported series of 234 eyes removed for ciliary body and choroidal melanomas.[11] Mäkitie et al.[9] have compared the utility of FVIII-RAg

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Supported by National Eye Institute Grant R01 EY10457 (RF) and Core Grant EY10792 (Department of Ophthalmology and Visual Sciences, University of Illinois at Chicago).

Submitted for publication January 4, 2002; revised March 14, 2002; accepted April 10, 2002.

Commercial relationships policy: N

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with CD34 in determining MVD in uveal melanoma and found similar results with each label, but indicate that CD34 results in a more distinct labeling and easier determination of MVD than does FVIII-RAg. Unstained sections from 214 of these tumors were available for staining with CD34 to determine MVD. Four-micrometer-thick sections were cut from each of these samples and stained for the presence of CD34 after a modification of the protocol established by Mäkitie et al.[9] tissue sections were stained for CD34QBend10 (Dako), the reaction product was detected by diaminobenzidine (DAB), and sections were counterstained by periodic acid-Schiff (PAS). After staining tissue sections, melanin was removed by bleaching with peroxide according to the method of Kivelä.[10] This procedural sequence does not interfere with antigenic detection in highly pigmented ocular tumors. In 14 of the 214 tumors available for study, immunohistochemistry was inadequate (e.g., CD34 failed to stain normal blood vessels adjacent to the tumor, the internal control). The final data set, therefore, consisted of 200 of the original 234 tumors in this series.

MVD was determined by modification of methods reported by Mäkitie et al.[9] Histologic sections stained for CD34 and developed with the DAB chromogen followed by bleaching to remove melanin, as described earlier, were examined at scanning magnification to identify three separate zones containing high numbers of discrete CD34 staining sites, and each of these hot-spot zones was photographed digitally. An area measuring 0.313 mm^2 was delimited on the digital photomicrograph. (Mäkitie et al.[9] counted $CD34^+$ structures directly from glass slides using the same area for each hot spot.) In each hot spot, each discrete focus of staining for CD34 was counted without requiring that a vessel lumen be present, in accordance with Weidner et al.[7] The pathologist photographing the hot spots and performing the counts was masked to outcome. The maximum MVD from the three hot spots measured was used in further statistical analyses.

Colocalization of S100 Protein and CD34 in Hot Spots

Histologic sections adjacent to those used to determine the maximum MVD from tissue sections stained with CD34 and labeled with DAB were double labeled for both S100 protein and CD34, with dual fluorescence used as described earlier. The slide stained only with CD34-DAB was used as a reference to identify the location of the hot spot on the adjacent section that was doubly labeled by immunofluorescence for CD34 and S100 protein. Each slide stained for both CD34 and S100 protein was examined under fluorescence, with a filter block used to detect only the $CD34^+$ signal (green). Thus, the pathologist taking these photomicrographs attempted to photograph structures that expressed CD34 without knowledge of any colabeling for S100 protein. From one to four digital photomicrographs were obtained from the green (CD34) channel, and, without moving the field, the filter block for the red (S100 protein) channel was rotated into position and the field photographed digitally. Negative control samples were examined to eliminate the possibility of autofluorescence.

Digital fluorescence photomicrographs were taken with the digital camera (Optronics), and the green and red channels were merged by the camera's software (MagnaFire software; Optronics). In each photomicrograph, at least five cells were analyzed for colocalization of S100 protein and CD34 by calculating the Pearson correlation coefficient for pixels expressing both red and green markers in the area of interest (ImagePro Plus software; Media Cybernetics, Silver Spring, MD). From each photograph, the highest of the Pearson correlation coefficients obtained from the five cells measured for colocalization was recorded. The pathologist who analyzed the digital immunofluorescence micrographs was different from the pathologist who identified the hot spots, measured MVD, and took the digital fluorescence micrographs. The pathologist who analyzed the micrographs was also masked to outcome.

Histologic Classifications

The data set of 234 uveal melanomas from which these 200 samples were drawn has been described previously.[11] In previous analyses of the data set of 234 tumors, the attribute of cell type was described as the presence of any epithelioid cells. Thus, tumors classified as mixed cell or epithelioid cell were collapsed into one category: epithelioid cells present. In the present study, cell type was stratified into two categories: spindle-mixed and epithelioid predominant.

Statistical Methods

To test for possible differences between the subset of 200 tumors used in this study and the larger series of 234 tumors, the distribution of tumor characteristics previously associated with outcome[11] was calculated for the subset and compared with the distribution in the overall data set by the Fisher exact test: largest tumor dimension (LTD; for this analysis it was stratified into three categories, $\leq$10, 10–15, and $>$15 microns), mitotic figures, cell type stratified on two levels as described earlier, patient's age (for this analysis, it was stratified into four categories, $<$50, 50–60, 60–70, $\geq$70 years), gender, and the presence or absence of PAS-positive loops, networks, parallel with cross or silent patterns. Cox proportional hazards models[12] were constructed for both data sets using death caused by metastatic uveal melanoma as the outcome.

The attribute reported as MVD ($CD34^+$ counts) was not normally distributed (Wilk-Shapiro[13] $W = 0.9313$, $P < 0.0001$). Transformed counts, obtained by taking the square root of MVD, were closer to but not quite normal (Wilk-Shapiro $W = 0.981092$, $P = 0.0085$). Hence, nonparametric tests were used in additional analyses.

To test for the effect of colocalization of S100 protein and CD34 on MVD, the squared Pearson correlation coefficient R^2 for the highest MVD hot spot for each case was grouped into classes: grade 0 ($R^2 < 0.6$), grade 1 (≥ 0.6, <0.85), grade 2 (≥ 0.85, <0.95), and grade 3 (≥ 0.95). The Pearson correlation coefficient was treated as a quantitative measure of colocalization intensity, similar to the pathologist's qualitative grades of absent (grade 0), weak (grade 1), moderate (grade 2), and intense (grade 3). Different cutoffs for the assignment of grade were tested throughout all analyses with no substantial differences in results.

The distribution of maximum MVD counts by colocalization grades was tested by the Kruskal-Wallis[14] χ^2 test. The relationship between maximum MVD count and colocalization grade was tested from another perspective: maximum MVD was divided into four groups, approximately corresponding to their quartiles. Association in the two-way table, colocalization grades versus maximum MVD, was tested using the contingency χ^2.

The influence of maximum MVD on outcome in this series was tested by the method of Kaplan-Meier. Cox proportional hazards models including maximum MVD and colocalization grades were developed. All software analyses were performed on computer (SAS software; SAS Institute, Cary, NC).

The research protocol, conforming to guidelines stipulated in the Declaration of Helsinki, was approved by Institutional Review Boards at the University of Iowa and the University of Illinois at Chicago.

Results

Labeling of Melanoma Cells by Endothelial Cell Markers

In preparation for a study of MVD in ciliary body and choroidal melanoma, tissue sections were stained with CD34 as recommended by Mäkitie et al.[9] However, after tissue sections were stained for CD34 and the stain was identified by the permanent chromogen DAB, all slides were subjected to peroxide bleaching to remove melanin,[10] and the tissues were lightly counterstained. It became apparent that some melanoma cells had labeled with CD34 (Fig 1A). Examination of the sections adjacent to those illustrated in Figure 1A revealed this tumor to be entirely amelanotic. Therefore, the brown intracytoplasmic material seen in Figure 1A was interpreted as positive staining for CD34.

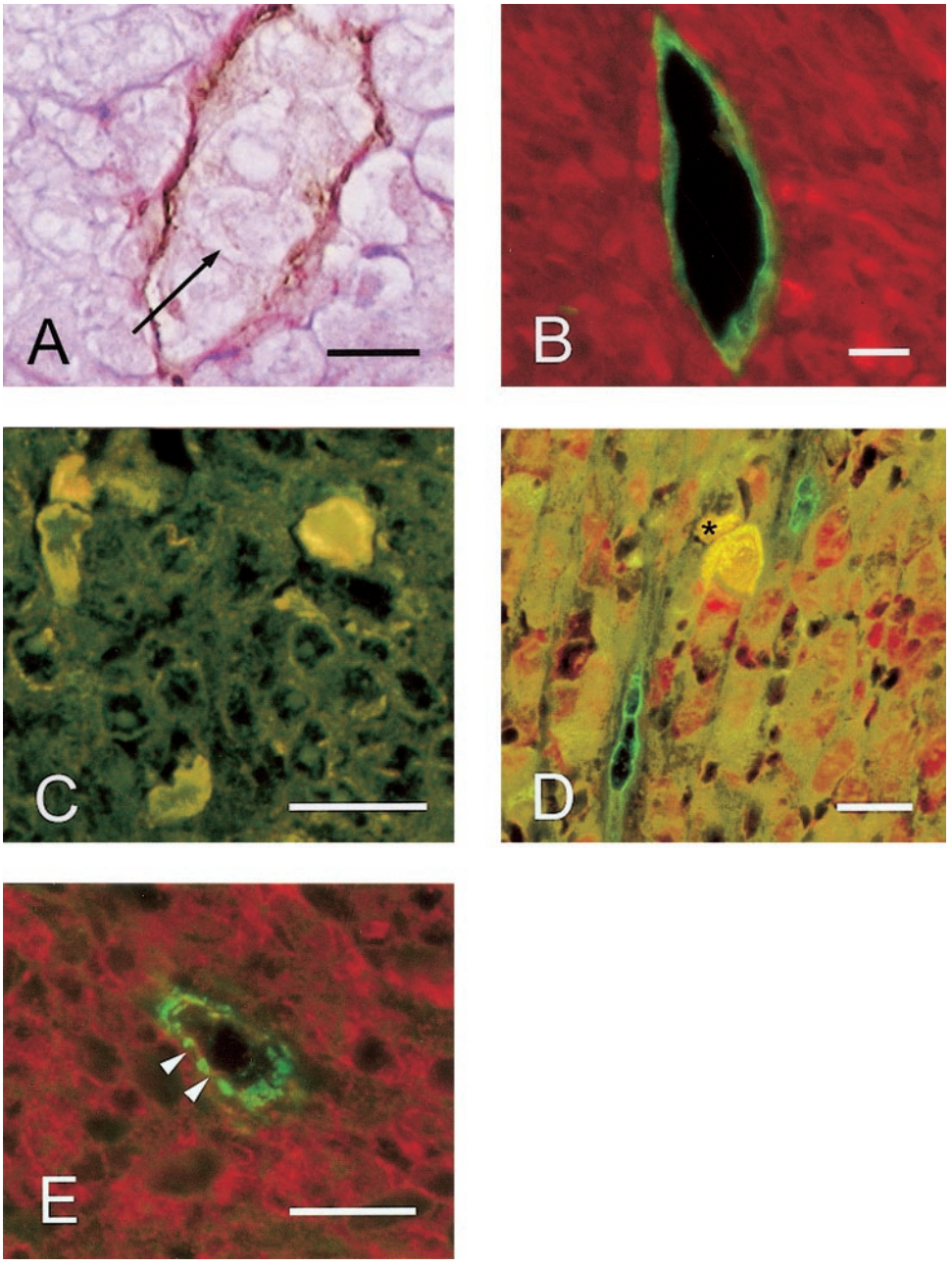

FIGURE 1. (**A**) A nest of epithelioid melanoma cells stained positively for CD34. Note the epithelioid melanoma cell positively stained for CD34 (*arrow*) and light staining of all tumor cells in this packet. This tumor was entirely amelanotic, and examination of the adjacent section revealed no melanin pigment within these cells. (**B**) Double immunofluorescence (S100 protein *red*, CD34 *green*): a large vessel in a choroidal melanoma is shown, lined entirely by $CD34^+$ cells. (**C**) Double immunofluorescence (Melan-A *red*, CD34 *green*): Melan-A and CD34 were strongly colocalized to tumor cells (*bright yellow*). Melan-A stained tended to stain far fewer tumor cells than did S100 protein (compare with **D**). (**D**) Double immunofluorescence (S100 protein *red*, CD34 *green*): two small vessels, lined entirely by CD34 (*green*) were identified, surrounded by tumor cells that expressed only S100 protein (*red*) and tumor cells that expressed both S100 protein and CD34 (*yellow*). (⋆)*Yellow* tumor cells analyzed by image analysis software. The Pearson correlation, the correlation of *red* and *green* pixels in this area of interest, was measured at $R^2 = 0.960529$. (**E**) Double immunofluorescence (S100 protein *red*, CD34 *green*): the endothelial ($CD34^+/S100^-$ cell lining was interrupted by the insertion of tumor cells ($CD34^-/S100^+$), forming a mosaic vessel. Note the presence of S100-positive (*red*) cytoplasm (*arrowheads*), indicating the presence of tumor cells between $CD34^+$ (*green*) endothelial cells. If endothelial cells had merely sloughed from these areas, the spaces between the endothelial cells would be vacant and not occupied by cells expressing the *red* chromogen S100 protein. Bars: (**A**) 10 μm; (**B–E**) 20 μm.

However, to validate the observation that some melanoma cells stained with CD34, tissue sections were double labeled for a marker associated with melanoma cells that is not expressed by endothelium. S100 protein is typically expressed in human uveal melanoma cells (uveal melanocytes are derived from neural crest) and not by vascular endothelium. S100 protein did not label the endothelium of choroidal vessels adjacent to the tumors. Melan-A is a marker that may be more specific for melanoma than S100 protein,[15,16] and similarly, no normal choroidal vessels stained with Melan-A. Despite reports of $CD34^+$ stromal cells in the dermis,[17] CD34 did not label stromal cells of the normal choroid or ciliary body. CD34 stained the endothelium of normal choroidal vessels adjacent to and within the tumors (Fig. 1B).

We discovered that S100 protein labeled tumor cells more diffusely than did Melan-A (compare Figs. 1C and 1D). Nevertheless, individual melanoma cells and clusters of melanoma cells were positive for both CD34 and Melan-A (Fig. 1C) and CD34 and S100 protein (Fig 1D).

In addition to vessels within melanomas lined exclusively by endothelium (Figs. 1B, 1D), intralesional vessels within the tumor were lined in part by $CD34^+$ and $S100^-$ cells (suggesting the presence of endothelium) and in part by tumor cells (Fig. 1E). The incorporation of tumor cells into the vessel wall has been described[18,19] and has recently been described as mosaic vessels.[19] The staining patterns of mosaic vessels (Fig 1E) were clearly different from small capillaries detected within the tumors (Fig. 1D) in which the lining of very small vessels labeled uniformly for CD34 exclusively.

Analysis of MVD

Cox proportional hazards models, calculated for the subset of 200 tumors available for this study were identical with that published previously for the entire data set of 234 tumors.[11]

TABLE 1. Cox Model

Data for 200 Eyes Removed with Ciliary Body or Choroidal Melanoma						
Characteristic*	**Parameter Estimate**	**Standard Error**	χ^2	***P***	**Hazard Ratio**	**95% Confidence Limits**
Network	0.70656	0.31791	4.9395	0.0263	2.027	1.087, 3.780
Mitoses	0.14479	0.03673	15.5395	<0.0001	1.156	1.976, 1.242
Largest tumor dimension	0.17298	0.04504	14.7533	0.0001	1.189	1.088, 1.299
Epithelioid cell type	1.24012	0.40749	9.2618	0.0023	3.456	1.555, 7.681
MVD-CD34	0.20765	0.07828	7.0363	0.0080	1.231	1.056, 1.435
Tumor lymphocytes	0.67419	0.28567	5.5698	0.0183	1.962	1.121, 3.435
Silent pattern	−0.75882	0.33809	5.0376	0.0248	0.468	0.241, 0.908

Summary of Stepwise Selection			
Step/Number in	**Characteristic***	**χ^2 Score**	***P***
1	Network	30.1965	<0.0001
2	Mitoses	14.9667	0.0001
3	Largest Tumor Dimension	8.5380	0.0035
4	Epithelioid cell type	13.1803	0.0003
5	MVD-CD34	6.8588	0.0088
6	Tumor lymphocytes	6.4600	0.0110
7	Silent pattern	5.1343	0.0235

* Largest tumor dimension expressed as largest dimension in contact with the sclera; MVD-CD34 expressed as square root transformation; tumor lymphocytes counted per 20 high-power field.[49]

The frequency distribution of tumor attributes from eyes in the subset of 200 tumors available for study with CD34 was not significantly different from the distribution of the larger data set by the Fisher exact test. MVD varied from tumor to tumor, as reported previously.[9] The median MVD based on the highest count among the three counts taken was 31/0.313 mm^2 (range, 7–105).

As reported previously by Mäkitie et al.,[9] the Kaplan-Meier analysis of survival for MVD-CD34 grouped by approximate quartiles was significant (χ^2 for the log-rank test was 16.9099, $P = 0.0007$). When MVD-CD34 are not grouped (expressed as MVD-CD34 without square root transformation) the univariate χ^2 for the log rank test was 9.6972 ($P < 0.0018$); and, when expressed as a square root transformation, the univariate χ^2 for the log-rank test was 12.1389 ($P < 0.0005$). Step-wise Cox models, developed to permit entry of the square root of MVD-CD34 in addition to previously reported tumor attributes of significance, yielded a final model that included both MVD-CD34 and networks (Table 1), similar to findings reported by Mäkitie et al.[9] It should be noted that for the square root of MVD-CD34, the hazard ratio and confidence interval 1.23 (1.06, 1.44) are almost exactly the same as that obtained by Mäkitie et al.

For colocalization R^2, the range is 0.3096 to 0.9911 (median, 0.8490). Table 2 summarizes the frequency distribution of tumor cells that were $CD34^+$ and $S100^+$ by grade of colocalization. No statistically significant correlations were shown between grade of colocalization and mitotic figures, epithelioid cell type, PAS-positive patterns, gender, LTD, or age.

TABLE 2. Frequency Distribution of $CD34^+$ Tumor Cells in Hot Spots by Grade of Colocalization

Colocalization Pearson Correlation Coefficient Squared	**Colocalization Grade**	**Number of Cases (%)**
<0.60	0	22 (11)
≥0.60, <0.85	1	78 (39)
≥0.85, <0.95	2	82 (41)
≥0.95	3	18 (9)
Total		200

Within hot spots, there was a significant relationship between the intensity grade of colocalization of S100 protein and CD34 and MVD. For example, of the 60 tumors in which MVD was 45 or more, 20 (34%) had grade 0 or 1 colocalization of CD34 and S100 protein, whereas 40 (66%) of 60 tumors in which MVD was 45 or more had grade 2 or 3 colocalization of CD34 and S100 protein (Table 3, $\chi^2 = 35.5049$, $P < 0.0004$; Fisher exact test $P = 0.0005$). These proportions were substantially different in the 44 tumors in which MVD was less than 20. Twenty-six (59%) tumors had grade 0 or 1 colocalization of CD34 and S100 protein, whereas 18 tumors (41%) had grade 2 or 3 colocalization of CD34 and S100 protein. Looking at the data from a different perspective, as the grade of colocalization increased, so did the median MVD (Table 4, Kruskal-Wallis $\chi^2=15.7322$, $P = 0.0013$).

When MVD was entered into the Cox models, the intensity of CD34/S100 colocalization did not appear in the model, either in terms of a grade (0–3) or as a continuous numeric variable.

DISCUSSION

In 1992, Weidner et al.[20] described the association between the density of vascularization in breast cancer and outcome. Staining sections of breast lesions for the presence of FVIII-RAg, Weidner et al.[20] advised scanning tissue sections to identify the zone of maximum concentration of marker staining. Each discreet focus of staining was to be counted as a separate vessel, even if the morphology suggested the same vessel snaking in and out of the section plane. The observations recorded by Weidner et al. in the breast were challenged subsequently.[21–24] Evidence both supporting[1] and challenging[2] the relationship of MVD to outcome in a variety of tumor systems has since been reported. The association between high MVD and metastasis has been postulated to represent either increased opportunities for tumor cells to enter the microcir-

TABLE 3. Distribution of CD34 Colocalization Grades across MVD-CD34$^+$ Levels

		MVD-CD34$^+$ (*n*)				
Correlation	**Grade**	**<20**	**20–30**	**>30–45**	**≥45**	**Total**
<0.6	0	4 (18, 9)*	12 (55, 26)	2 (9, 4)	4 (18, 7)	22
>0.6, <0.85	1	22 (28, 50)	22 (28, 48)	18 (23, 36)	16 (21, 27)	78
≥0.8, <0.95	2	17 (21, 39)	9 (11, 20)	25 (30, 50)	31 (38, 51)	82
≥0.95	3	1 (6, 2)	3 (17, 6)	5 (28, 10)	9 (50, 15)	18
Total		44 (22)	46 (23)	50 (25)	60 (30)	200

Table $\chi^2 = 30.5049$, $P = 0.0004$; Fisher exact test $P = 0.0005$.

* Data in parentheses are percentage of row total (total for correlation shown) and percentage of column total (total for MVD shown). Data in parentheses are percentage of the total sample ($n = 200$).

culation through increased exposure to microvessels or to reflect increased angiogenesis by aggressive cancers (i.e., high MVD is an indirect measure of tumor aggressiveness).[25]

One would have suspected that the association between MVD and outcome would have been confirmed easily in uveal melanoma, because unlike breast cancer or cutaneous melanoma, which may spread by either lymphatics or by the blood stream, uveal melanoma spreads exclusively by the hematogenous route, because there are no lymphatics within the eye or within uveal melanomas.[26] Nevertheless, Lane et al.[27] and Schaling et al.[28] both failed to discover an association between MVD and outcome. Foss et al.,[8] using FVIII-RAg to label tissue sections of uveal melanomas and the techniques advocated by Weidner et al.[20] for locating hot spots, discovered an association between MVD and adverse outcome but failed to confirm previous observations[11] that PAS-positive looping patterns contribute independently to death in metastatic melanoma.

Mäkitie et al.[9] confirmed the association of both MVD and PAS-positive patterning in choroidal and ciliary body melanoma and death caused by metastatic disease. These investigators pointed out that neither Lane et al.[27] nor Schaling et al.,[28] who failed to discover associations between MVD and outcome, used the method suggested by Weidner et al.[20] Mäkitie et al.[9] also postulated that Foss et al.[8] defined PAS-positive patterns in a fashion different from that used by Folberg et al.[11] and by subsequent groups[29–31] who confirmed the association between these patterns and outcome: The distribution of looping PAS-positive patterns in the study by Foss et al.[8] varied from that described by those who found these patterns to be prognostically significant.

After the publication of the work by Mäkitie et al.[9] indicating an independent effect of both MVD and PAS-positive patterns on mortality in ciliary and choroidal melanoma, attention was drawn to the observation that aggressive uveal melanoma cells appear to be genetically deregulated and may express markers not typical for melanoma cells or cells derived from neural crest.[32–34] Moreover, the specificity of endothelial cell markers has long been called into question. Mäkitie et al.[9] preferred CD34 to FVIII-RAG in studies of MVD in uveal melanoma. Although the influence of FVIII-RAg staining on MVD was not examined in the present study, FVIII-RAg is not a specific marker for endothelium. The cell line ECV-304 which was originally reported to be a human immortalized vascular endothelial cell (HUVEC) line by virtue of expression of factor VIII, ultrastructural features such as Weibel-Palade bodies, and the formation of tubules on synthetic basement membrane,[35,36] was recently discovered to be a derivative of the human bladder tumor cell line T14.[37] CD34 is a 115-kDa cell surface protein expressed by myeloid and lymphoid progenitor cells, and it has been observed that CD34 may be expressed in endothelial cells during angiogenesis.[38] However, CD34 is also present in lesions derived from the neural crest, such as neurofibromas,[39] cellular blue nevi,[6] and desmoplastic cutaneous melanoma.[4] In some cellular blue nevi, there is diffuse staining of melanocytes by CD34,[6] consistent with our observation of diffuse expression of CD34 in tumor cells in some uveal melanomas (Figs. 1C, 1D).

TABLE 4. Distribution Of Median MVD per Grade of CD34/S100 Protein Colocalization

Colocalization Grade	**Cases (*n*)**	**Median MVD**
0	22	25.0
1	78	27.0
2	82	35.5
3	18	42.0

Kruskal-Wallis $\chi^2 = 14.5853$; $P = 0.0222$.

In this study, both MVD and PAS-positive patterns exerted independent effects on death caused by ciliary body and choroidal melanoma, as demonstrated by Mäkitie et al.[9] The Cox model developed from our data (Table 1) differs from previously published models from this data set,[11] in that previously, our classification pertaining to cell type was dichotomous (epithelioid cells absent or present) and cell type did not enter the final model, whereas in the present study, our dichotomous classification was epithelioid predominant or not. With this modification in classification, cell type (epithelioid cell predominant) was entered into the model (Table 1), and the "silent" pattern was also entered into the model but with a hazard ratio less than 1, indicating a protective effect by the presence of this feature. Indeed, the silent pattern represents foci in which neither blood vessels nor PAS-positive patterns are present,[40] consistent with improved survival.

In this study, we also investigated the possibility that uveal melanoma cells may label for markers used in the assessment of MVD. Folberg et al.[2] have already shown that the nuclear matrix of some choroidal and ciliary body melanoma cells labels with CD31, but CD31 has not been used in studies of MVD in uveal melanoma. In histologic sections of choroidal and ciliary body melanoma, we identified epithelioid melanoma cells that stained for CD34 (Fig. 1A). However, to eliminate confusion introduced by the subjective assignment of cells according to histologic lineage, we investigated the labeling of melanoma cells by CD34 by labeling tissue sections for markers expressed in melanoma cells but not on endothelial cells and double labeling for the endothelial cell marker of interest, CD34. S100 protein is a nonspecific marker associated with cells of neural crest lineage, and therefore not expressed on vascular endothelium. HMB45, Melan-A, and micro-ophthalmia transcription factor (MiTF) are more specific for melanoma than S100 protein in the detection of uveal melanoma cells.[16,41]

S100 protein is diffusely distributed in uveal melanomas, but is less specific a marker for melanoma cells than MiTF, HMB45, or Melan-A. MiTF, however, is a related to differentiation, and expression of MiTF in cutaneous melanoma has been shown to be associated with a favorable outcome.[42] In preliminary studies in our laboratory, MiTF was not diffusely expressed by melanoma cells in aggressive tumors and was not studied further as a marker for melanoma cells in this study. The distribution of HMB45 and Melan-A in ocular melanocytic lesions has been studied by Heegaard et al.[16] and was found to be comparable to the distribution of S100 protein in melanoma cells.

In exploratory studies, we discovered that S100 protein was more diffusely distributed through the lesion than the other markers, and S100 protein was used to explore the possibility that melanoma cells that express an endothelial cell marker contribute to the calculation of MVD in a hot spot in our series of 200 melanomas. To mirror the study by Mäkitie et al.[9] as closely as possible, we used CD34 as a marker for endothelium in our study of MVD. Mäkitie et al.[9] had demonstrated no significant difference between the calculation of MVD from CD34-stained sections and sections stained for FVIII-RAg, but found that CD34 rendered a reaction product that was easier to interpret. It is noteworthy that not only did melanoma cells colabel for S100 protein and CD34, but also with Melan-A and CD34.

In this study, we graded the coexpression (absent, weak, moderate, intense) of S100 protein and CD34 based on the Pearson correlation coefficient for colocalization within the cell of interest. This may represent too stringent a definition for colocalization. We and our colleagues[32–34,43] have shown that aggressive melanoma cells are genetically deregulated cells and that they express inappropriate markers not expected of cells of melanocytic lineage. It is therefore possible that the deregulated melanoma cells may lose expression of S100 protein or Melan-A as they acquire expression of CD34. For example, the expression of MiTF, a melanoma marker, appears to be a marker of differentiation, and there is a tendency to observe reduced expression of MiTF in more aggressive cutaneous melanomas.[42]

Nevertheless, by requiring that a cell coexpress a melanoma marker along with CD34, we achieve some measure of certainty that the cell labeling is not an endothelial cell. Not only does CD34 label melanoma cells in uveal melanoma hot spots (Figs. 1C, 1D), but there also appears to be an association between MVD and the presence of $CD34^+$ melanoma cells in the hot spot (Tables 3, 4). Thus, in uveal melanomas, the characteristic known as MVD is not a pure quantification of blood vessels per unit area, but is rather a hybrid measurement of blood vessels and melanoma cells.

It is intriguing to speculate that the inappropriate expression of CD34 on melanoma cells is a marker of aggressive behavior. The observation that there are significantly fewer $CD34^+$ melanoma cells in hot spots with low MVD than in those with high MVD (Tables 3, 4) suggests that the number of $CD34^+$ melanoma cells may be a risk factor for metastasis, but we did not quantify the number of $S100^+$ tumor cells labeled with CD34. In this study, the mere presence of strongly positive $CD34^+$ melanoma cells in hot spots was not itself associated with death caused by metastatic melanoma.

If high MVD is not necessarily equivalent to a hot spot of vascularity, then it is reasonable to challenge the assumption that high MVD reflects increased angiogenesis. Indeed, in this study results showed that the blood supply to uveal melanomas was complex and heterogeneous. Aside from normal vessels incorporated into the tumor (Fig. 1B) and vessels within the tumor originating from a presumed angiogenic response (Fig. 1D), some vessels are lined in part by endothelial cells and in part by tumor cells, consistent with mosaic vessels[19] (Fig. 1E). The incorporation of tumor cells into tumor vessels has been well established.[18,44–46] In animal models of colon cancer and in human colon cancer specimens, it has been estimated that 13% of tumor vessels are mosaic (contain endothelial cells and tumor cells), representing 4% of the total vascular volume of microcirculation in these tumors. Folkman[47] has suggested that the incorporation of tumor cells into blood vessels (vascular mosaicism) may be a mechanism that enhances metastasis by the shedding of tumor cells into the microcirculation. Finally, in addition to circulation within blood vessels, it is possible that blood and plasma may circulate within components of highly patterned extracellular matrix that is generated by the tumor cells themselves.[2,32] The contribution of patterned matrix to a functional circulation (vasculogenic mimicry) is unclear and controversial.[48]

In this study MVD, as defined by Weidner et al.[20] and modified for use in uveal melanoma by Mäkitie et al.,[9] was independently associated with death caused by metastatic melanoma and that PAS-positive looping patterns enter the Cox model along with MVD. However, the colocalization of the putative endothelial cell marker CD34 to melanoma cells in hot spots measured for the calculation of MVD suggests strongly that MVD cannot be equated with angiogenesis. These findings, if replicated with other markers and in other tumor systems, may have important implications for pathologists who associate MVD with outcome and investigators who rely on the associations between MVD and tumor behavior when designing new therapies to target angiogenesis.

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