Tumor-Infiltrating Macrophages (CD68+ Cells) and Prognosis in Malignant Uveal Melanoma

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PURPOSE. To investigate the hypothesis that tumor-infiltrating macrophages contribute to prognosis of uveal melanoma and to study their association with tumor characteristics, especially microvessels.

METHODS. This was a retrospective, population-based cohort study of 167 consecutive patients who had had an eye with choroidal or ciliary body melanoma removed between 1972 and 1981. Macrophages were identified with mAb PG-M1 to the CD68 epitope, and their number and morphologic type were recorded. Kaplan-Meier and Cox regression analyses of melanoma-specific survival were performed.

RESULTS. CD68-positive macrophages could be assessed in 139 (83%) of the 167 melanomas. Their number was moderate to high in 115 (83%) of the 139 tumors, and their morphology ranged from dendritic to round. A high number of macrophages was associated with presence of epithelioid cells (P = 0.025), heavy pigmentation (P = 0.001), and high microvascular density (P = 0.001). The 10-year melanoma-specific mortality rate increased with higher numbers of macrophages (0.10 for low versus 0.57 for high numbers, P = 0.0012). The morphologic type of infiltrating macrophages was not associated with mortality. The number of macrophages was modeled by stratification, which significantly improved a Cox regression model (P < 0.001). Adjusting for the other independent indicators of metastatic death 10-year melanoma-specific mortality was 0.17 for low versus 0.45 for high numbers of macrophages.

CONCLUSIONS. The number of tumor-infiltrating CD68-positive macrophages contributes to prognosis and associates with cell type and microvascular density, which merits a further analysis of the biological role of these cells in uveal melanoma. (Invest Ophthalmol Vis Sci. 2001;42:1414–1421)

Clinical and experimental evidence has been provided to support the theory that tumor cells of malignant uveal melanoma, a cancer that can spread only hematogenously, because the eye and orbit have no lymphatic vessels, may be able to generate nonconventional microvascular channels without active contribution of endothelial cells.1,2 Those melanomas that behave aggressively and often metastasize display microvascular loops and networks surrounding nests of tumor cells.3,4 This proposed form of vasculogenesis is tentatively called microvascular mimicry.1,2 The pathophysiologic significance and clinical importance of microvascular mimicry compared with conventional angiogenesis is still open to heated debate.2,5–7 Irrespective of how these microvessels form, the patterns they generate have been shown to be an independent prognostic factor that predicts a high chance of metastasis.8–10 On balance, high microvascular density (MVD), evaluated in areas of densest vascularization, a rough quantitative measure of tumor microvessels that has been linked with angiogenesis in certain cancers,11 also independently contributes to risk of metastasis in uveal melanoma.12,13

The number of non-neoplastic stromal cells such as macrophages may sometimes be of the same order of magnitude as the number of tumor cells.14 The number of macrophages correlates with MVD in cutaneous melanoma, breast carcinoma, and non-Hodgkin’s lymphoma.15–17 We recently noted that uveal melanomas including non-necrotic tumors, treated with simple enucleation, contain notable numbers of macrophages,18 and we designed the present study to assess the extent and type of macrophage infiltration in a consecutive and population-based series of patients. Our specific purpose was to determine whether infiltrating macrophages carry prognostic information and whether they are associated with other characteristics of the tumor, especially its microvascular properties.

PATIENTS AND METHODS

Study Population and Exclusion Criteria

One hundred sixty-seven consecutive patients who had had an eye with choroidal or ciliary body melanoma removed between 1972 and 1981 were ascertained from the diary of the Ophthalmic Pathology Laboratory, Helsinki University Central Hospital, as previously described.10 During that period, prompt enucleation was the standard treatment for all but the smallest uveal melanomas, which were first observed for growth. All eyes enucleated in the district were routinely submitted to the Ophthalmic Pathology Laboratory, making the series essentially unselected and representative of all malignant uveal melanomas treated during that period.

Follow-up data for this cohort of patients was updated to December 1999 by previously described routines.10 The median follow-up time was 22 years (range, 18–26). Altogether, 50 (53%) of 80 deaths caused by uveal melanoma and all 9 deaths caused by other cancers were reconfirmed by immunohistochemistry on biopsy specimens or specimens obtained at autopsy.10 In addition, 14 deaths of melanoma had been confirmed by fine-needle aspiration biopsy.

Specimens in which less than 50% of the original melanoma remained (14 patients), the remaining tumor was entirely above Bruch’s membrane (16 patients), or the block was missing (2 patients) were excluded from the present analysis, leaving 149 specimens to be studied (inclusion rate, 89%). Unlike the criteria used in our previous analysis of microvascular patterns and density,10,13 the current criteria
did not exclude specimens that were more than 50% necrotic (15 patients), so that we could determine to what extent tumor necrosis was associated with the overall number of infiltrating macrophages.

**Immunoperoxidase Staining**

The paraffin blocks were cut at 5 μm, after which the slides were randomly coded by an outside laboratory technician. The code was broken only after macrophage and survival data were ready for analysis, with all investigators masked to the outcome of individual patients until that time. Immunostaining of macrophages was performed using the avidin-biotinylated peroxidase complex method (Vectastain ABC Elite Kit; Vector Laboratories, Burlingame, CA), as described previously in detail.18

Two primary mouse monoclonal antibodies (mAbs) PG-M1 (IgG; lot 101, diluted 1:50; Dakopatts, Klostrup, Denmark) and KP1 (IgG; lot 058, diluted 1:100; Dakopatts) were used to recognize fixative-resistant epitopes on CD68, an intracytoplasmic 110-kDa glycoprotein that resides in lysosomal granules and is expressed by macrophages in all human tissues.19,20 A third mAb, clone 3A5 against human macrophages (IgG2b; lot 210501, diluted 1:50; Novocastra Laboratories, Newcastle-upon-Tyne, UK), was also used. Pretreatment with 0.4% (wt/vol) pepsin (FIP, 2500 U/g; E. Merck, Darmstadt, Germany) in 0.01 M hydrochloric acid for 15 minutes at 37°C enhanced immunostaining with mAb PG-M1, and pretreatment in 10 mM sodium citrate buffer (pH 6.0) for 10 minutes at 95°C enhanced immunostaining with mAbs KP1 and 3A5. In preliminary experiments, mAb 3A5 immunolabeled dendritic macrophages less effectively than mAb PG-M1, in accordance with a previous study,21 and mAb KP1 labeled fewer macrophages overall than mAb PG-M1 did. mAb KP1 also cross-reacted with melanoma cells in several tumors, as has been noted earlier in cutaneous melanoma.19,22 Consequently, mAb PG-M1 was used throughout this study as the default antibody to quantitate macrophages. The primary mAb QBEND/10 (lot 121202, diluted 1:25; Novocastra) to the CD54 epitope of endothelial cells was used to label microvessels.3

To enable evaluation of immunoreaction in pigmented tumors, the peroxidase reaction was developed with 3,3′-diaminobenzidine tetrahydrochloride and, regardless of the grade of pigmentation, melanin was then bleached with 3.0% (vol/vol) hydrogen peroxide and 1.0% hydrochloric acid for 15 minutes at 37°C. MVD was divided in quartiles.13

### Grading of Infiltrating Macrophages

To develop a repeatable grading system that could be used by other laboratories as well, specimens of uveal melanomas that were not included in the study series were immunostained with mAb PG-M1. From this group of specimens it was obvious that immunolabeled cells differed not only in number but also in morphologic appearance, and ranged from dendritic to round phagocytosing cells.

The pilot set of specimens was divided into three groups based on the overall number of cells immunopositive with mAb PG-M1 in non-necrotic areas of the tumor. Confluent necrotic areas did not influence the grading. Whereas round immunopositive cells were relatively easy to count, it was not possible to determine the number of dendritic cells reliably. For this reason, we graded the number of immunopositive cells semiquantitatively. Standard photographs that represented few (Figs. 1A, 1B), moderate number of (Figs. 1C, 1D), and many immunopositive cells (Figs. 1E–G) were taken.

The tumors were similarly semiquantitatively divided in three groups based on the predominant type of cells immunoreactive with mAb PG-M1. Standard photographs were taken that represented tumors in which the majority (75% or more) of immunopositive cells were either dendritic (Figs. 1A, 1C, 1E) or round (Figs. 1B, 1D, 1F). The third group consisted of tumors in which neither dendritic nor round immunopositive cells clearly predominated or the morphology of immunopositive cells was intermediate between dendritic and round (Fig. 1G). Confluent immunopositive cells in necrotic areas did not influence the grading.

Melanomas in the study series were subsequently graded independently by two observers under a light microscope based on the overall number of immunopositive cells and their predominant morphologic type, according to the standard photographs (Figs. 1A–G). Discrepancies in grading were resolved by consensus under a double-headed microscope.

### Grading of Microvessels

Microvascular loops and networks, consisting of at least three back-to-back loops, were identified under a green filter according to the criteria of Folberg et al.5,4 from sections first bleached with potassium permanganate and oxalic acid and then stained with acid Schiff without counterstain, as described previously in detail.10 MVD was assessed in the most highly vascularized area using an eyepiece with an etched graticule corresponding to 0.313 mm² at ×200 magnification (WK 10×/20L-H; Olympus, Tokyo, Japan).12,15 Any immunolabeled vessel that was clearly separate from an adjacent one and was either totally inside the graticule or touching its top or left border was counted as a microvessel.

### Statistical Analysis

Analyses were performed by computer (PC-90 software; BMDP Statistical Software, Cork, Ireland). Exact probability distributions were also computed (StatXact-3; Cytel Software, Cambridge, MA). Fisher’s exact and Pearson’s χ² tests were used to compare proportions in unordered contingency tables, and Kruskal-Wallis and Jonckheere-Terpstra tests were used to compare proportions in singly and doubly ordered contingency tables, respectively.23,21 p < 0.05 was considered statistically significant. The weighted k statistic was used to estimate chance-corrected interobserver agreement in grading the number and type of macrophages.24

The number of macrophages was analyzed as an ordered (few, moderate, many) CD68-immunopositive cells) and the predominant morphologic type of macrophages as an unordered three-category variable (dendritic, intermediate, round CD68-immunopositive cells). Cell type was collapsed into two categories according to the presence or absence of epithelioid cells (spindle, nonspindle) and tumor location according to the presence or absence of ciliary body involvement.4,10 Largest basal tumor diameter (LBD) was divided in three categories according to the size of the tumor (<10 mm, 10–15 mm, >15 mm).4,11 Degree of pigmentation in each tumor was graded semiquantitatively by sorting unstained 5-μm-thick paraffin-embedded sections into three groups that represented amelanotic to weak, moderate, and strong pigmentation. Microvascular loops and networks were analyzed as a combined variable that considered networks to be an advanced stage of loops (no loops, loops without networks, networks).10 MVD was divided in quartiles.13

Univariate analysis of survival time data were based on the Kaplan-Meier product-limit method,22 and a trend version of this test was used if the categories analyzed were ordered. In pair-wise comparisons, probabilities were adjusted according to Bonferroni.24 Patients judged to die of causes unrelated to uveal melanoma were censored at the time of death. Equality of follow-up between groups was ascertained by comparing Kaplan-Meier curves with reverse censoring.25

Because no previous study on macrophage infiltration and survival in uveal melanoma was available, formal sample-size calculation for the Kaplan-Meier analysis was not feasible. Power analysis by simulation26 indicated that the present study had 80% power to detect a 0.25 difference in 20-year survival (or a hazard ratio of 1.9), and 95% power to detect a 0.32 difference in survival (or a hazard ratio of 2.4).

Multivariate analysis of melanoma-specific survival was based on the Cox proportional hazards model.25,27 The assumption of proportional hazards was assessed by adding each covariate by log time interaction to the model and assessing the significance of the product term using the partial likelihood ratio test.27 LBD and MVD were analyzed as continuous variables, the latter using square root-transformed counts, which rendered it normally distributed.12,15 The three unordered categories of the type of macrophages were modeled with two design variables.27 A maineffects model was adjusted for the effect of tumor pigmentation and macrophages.13 The number of variables in
the final model was restricted to four, based on a rule that requires at least 15 to 20 events per variable. The regression coefficients and hazard ratios (HRs) with 95% confidence intervals (CIs) were calculated.

RESULTS

The clinical and histopathologic characteristics of the 149 tumors included in and the 28 tumors excluded from the present study were comparable in tumor location ($P = 0.23$, Fisher’s exact test), presence of epithelioid cells ($P = 0.30$), and LBD ($P = 0.072$, Mann-Whitney test).

Number and Type of Macrophages

Immunostaining with mAb PG-M1 to the CD68 epitope was satisfactory in 139 (93%) of the 149 specimens of uveal melanoma studied. Immunopositive cells were easily recognizable and mainly infiltrated diffusely (Figs. 1A–G), but in some areas they arranged roughly along microvascular loops, networks, and other microvascular patterns (Fig. 1H). An area in which immunopositive cells align along a microvascular loop (H). Immunoperoxidase staining with mAb PG-M1 and bleaching of melanin. Individual immunopositive cells could not be counted reliably (e.g., E, F). Photographs (B), (D), and (F) show areas of approximately 130, 360, and 650 immunopositive cells/mm². Scale bars, 100 µm.

After consensus, the number of CD68-immunopositive cells was semiquantitatively graded as few in 24 tumors (17%; 95% CI, 11–25), moderate in 71 tumors (51%; 95% CI, 43–60), and many in 44 tumors (32%; 95% CI, 24–40). The predominant type of infiltrating cells was graded as dendritic type in 30 tumors (22%; 95% CI, 15–29), intermediate in 82 tumors (59%; 95% CI, 50–67), and round in 27 tumors (19%; 95% CI, 13–27).

Interobserver Agreement

The two investigators agreed on the number of CD68-immunopositive cells in 80% (95% CI, 72–86) of specimens. No two-category discrepancies occurred. They agreed on the predominant type of CD68-immunopositive cells in 67% (95% CI, 58–75) of specimens, and solved a discrepancy of two catego-
Microvascular patterns

No loops

Dendritic

Intermediate

Round

CD68+ cells

Necrosis

< 10 mm

10–15 mm

15 mm

Cell type

Spindle

Nonspindle

Necrosis

Less than 50%

More than 50%

Tumor location

Choroid only

Ciliary body involved

LBD

Melanomas without epithelioid cells, and melanomas with low MVD, respectively, but overlap between categories was noted (Figs. 2A, 2B). In addition, females had significantly more immunopositive cells than males (P = 0.010, Kruskal-Wallis test).

No association between the predominant type of CD68-immunopositive cells and LBD, the presence of epithelioid cells, MVD, and gender was observed (Table 1). Weakly pigmented tumors (50%–57 microvessels) 0.001, Jonckheere-Terpstra test), heavy pigmentation (P

Neither the number nor the predominant type of immunopositive cells was significantly associated with involvement of the ciliary body, presence of microvascular loops and networks, presence of extracocular extension, and presence of more than 50% necrosis (Table 1).

The number of CD68-immunopositive cells was significantly associated with four known prognostic indicators (Table 1). Melanomas with large basal diameter (P = 0.031, Jonckheere-Terpstra test), heavy pigmentation (P = 0.001), epithelioid cells (P = 0.025, Kruskal-Wallis test), and high MVD (P = 0.001, Jonckheere-Terpstra test) had significantly more immunopositive cells than small and weakly pigmented melanomas, melanomas without epithelioid cells, and melanomas with low MVD, respectively, but overlap between categories was noted (Figs. 2A, 2B). In addition, females had significantly more immunopositive cells than males (P = 0.010, Kruskal-Wallis test).

The interobserver agreement (weighted k) was 0.77 (95% CI, 0.69–0.85) for grading the number and 0.60 (95% CI, 0.49–0.71) for grading the type of CD68-immunopositive cells.

**Associations with Other Variables**

The associations with other variables are listed in **Table 1**.

**Table 1.** The Association between Clinical and Histopathologic Characteristics, Number, and Type of Cells Immunopositive with mAb PG-M1 to the CD68 Epitope of Macrophages in 139 Patients with Choroidal and Ciliary Body Melanoma

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Few</th>
<th>Moderate</th>
<th>Many</th>
<th>P</th>
<th>Dendritic</th>
<th>Intermediate</th>
<th>Round</th>
<th>P</th>
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<tr>
<td>Male</td>
<td>16 (67)</td>
<td>30 (42)</td>
<td>14 (32)</td>
<td>0.010*</td>
<td>15 (50)</td>
<td>34 (41)</td>
<td>11 (41)</td>
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<td>Female</td>
<td>8 (33)</td>
<td>41 (58)</td>
<td>30 (68)</td>
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<td>15 (50)</td>
<td>48 (59)</td>
<td>16 (59)</td>
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<td>Choroid only</td>
<td>20 (85)</td>
<td>51 (75)</td>
<td>51 (70)</td>
<td>0.32*</td>
<td>25 (85)</td>
<td>57 (70)</td>
<td>20 (74)</td>
<td>0.41†</td>
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<td>Ciliary body involved</td>
<td>4 (17)</td>
<td>19 (27)</td>
<td>13 (30)</td>
<td></td>
<td>5 (17)</td>
<td>24 (30)</td>
<td>7 (26)</td>
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<td>LBD ≤ 10 mm</td>
<td>12 (50)</td>
<td>19 (27)</td>
<td>10 (23)</td>
<td>0.031†</td>
<td>6 (20)</td>
<td>31 (38)</td>
<td>4 (15)</td>
<td>0.55*</td>
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<td>&gt; 10–15 mm</td>
<td>9 (38)</td>
<td>32 (45)</td>
<td>20 (45)</td>
<td></td>
<td>16 (55)</td>
<td>33 (40)</td>
<td>12 (44)</td>
<td></td>
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<tr>
<td>&gt; 15 mm</td>
<td>3 (12)</td>
<td>20 (28)</td>
<td>14 (32)</td>
<td></td>
<td>8 (27)</td>
<td>18 (22)</td>
<td>11 (41)</td>
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<td><strong>Cell type</strong></td>
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<tr>
<td>Spindle</td>
<td>20 (87)</td>
<td>39 (60)</td>
<td>23 (55)</td>
<td>0.025*</td>
<td>19 (65)</td>
<td>45 (60)</td>
<td>18 (72)</td>
<td>0.56†</td>
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<td>Nonspindle</td>
<td>3 (13)</td>
<td>26 (40)</td>
<td>19 (45)</td>
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<td>11 (37)</td>
<td>30 (40)</td>
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<td><strong>Necrosis</strong></td>
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<tr>
<td>Less than 50%</td>
<td>25 (96)</td>
<td>65 (92)</td>
<td>42 (95)</td>
<td>0.90*</td>
<td>30 (100)</td>
<td>75 (91)</td>
<td>25 (93)</td>
<td>0.29†</td>
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<td>More than 50%</td>
<td>1 (4)</td>
<td>6 (8)</td>
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<td>7 (9)</td>
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<td><strong>Tumor pigmentation</strong></td>
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<tr>
<td>Weak</td>
<td>16 (67)</td>
<td>19 (27)</td>
<td>2 (4)</td>
<td>0.001†</td>
<td>20 (67)</td>
<td>17 (21)</td>
<td>0 (0)</td>
<td>0.001*</td>
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<tr>
<td>Moderate</td>
<td>6 (25)</td>
<td>36 (51)</td>
<td>25 (57)</td>
<td></td>
<td>9 (30)</td>
<td>48 (58)</td>
<td>10 (37)</td>
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<tr>
<td>Strong</td>
<td>2 (8)</td>
<td>16 (22)</td>
<td>17 (39)</td>
<td></td>
<td>1 (3)</td>
<td>17 (21)</td>
<td>17 (63)</td>
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<td><strong>CD68+ cells</strong></td>
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<td>Dendritic</td>
<td>9 (38)</td>
<td>15 (21)</td>
<td>6 (14)</td>
<td>0.005*</td>
<td>N/A</td>
<td>N/A</td>
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<td>Intermediate</td>
<td>13 (54)</td>
<td>46 (65)</td>
<td>23 (52)</td>
<td></td>
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<td>N/A</td>
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<tr>
<td>Round</td>
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<td>10 (14)</td>
<td>15 (34)</td>
<td></td>
<td>N/A</td>
<td>N/A</td>
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<td><strong>Microvascular patterns</strong></td>
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<tr>
<td>No loops</td>
<td>12 (52)</td>
<td>21 (32)</td>
<td>20 (47)</td>
<td>0.64‡</td>
<td>12 (40)</td>
<td>26 (35)</td>
<td>15 (60)</td>
<td>0.34*</td>
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<td>Loops only</td>
<td>7 (31)</td>
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<td>9 (30)</td>
<td>19 (25)</td>
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<td>Networks</td>
<td>4 (17)</td>
<td>26 (40)</td>
<td>15 (36)</td>
<td></td>
<td>9 (30)</td>
<td>30 (40)</td>
<td>6 (24)</td>
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<td>Microvascular density</td>
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<tr>
<td>&lt; 25 microvessels</td>
<td>9 (39)</td>
<td>19 (29)</td>
<td>6 (14)</td>
<td>0.001‡</td>
<td>7 (25)</td>
<td>18 (24)</td>
<td>9 (36)</td>
<td>0.44*</td>
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<td>25–40 microvessels</td>
<td>9 (39)</td>
<td>17 (26)</td>
<td>7 (17)</td>
<td></td>
<td>9 (30)</td>
<td>21 (28)</td>
<td>3 (12)</td>
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<td>41–57 microvessels</td>
<td>3 (13)</td>
<td>14 (22)</td>
<td>14 (35)</td>
<td></td>
<td>6 (20)</td>
<td>18 (24)</td>
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<tr>
<td>&gt; 57 microvessels</td>
<td>2 (9)</td>
<td>15 (23)</td>
<td>15 (36)</td>
<td></td>
<td>8 (27)</td>
<td>18 (24)</td>
<td>6 (24)</td>
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</tr>
</tbody>
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Data are number of patients with percentage of total for each parameter in parentheses. N/A, not applicable.

* Two-sided Kruskal-Wallis test.
† Two-sided χ² test for independence.
‡ Two-sided Jonckheere-Terpstra test.
§ Single globally highest vessel count per 0.313 mm² area obtained with antibody QBEND/10 to the CD34 epitope.
Survival differed by 0.28 between patients with high and low numbers of macrophages. The difference in survival slightly decreased over long follow-up time (Fig. 3).

DISCUSSION

It has long been recognized that uveal melanomas contain macrophages, in particular melanophages, but this type of tumor-infiltrating cell has neither been studied in great detail nor has it been depicted as a major and ubiquitous component of uveal melanoma. In the ongoing Collaborative Ocular Melanoma Study (COMS), 1354 (89%) of 1526 enucleated melanomas had “none to minimal” or “scattered melanomas had “none to minimal” or “scattered single small clumps,” and 172 (11%) tumors had “scattered single and larger aggregates” of macrophages by light microscopy. Using immunohistochemistry with mAb PG-M1 to the CD68 epitope 19 and bleaching of melanin, we found the predominant type of macrophages and tumor pigmentation did not enter the model. This model was strongly preferred to the nonstratified model that disregarded the number of macrophages (likelihood ratio = 245.1–227.7, $P < 0.001$ $\chi^2$ test, 1 df, indicating that the stratified model predicted melanoma-specific mortality more precisely than the nonstratified model). After adjustment, the melanoma-specific 10-year survival differed by 0.28 between patients with high and low numbers of macrophages. The difference in survival slightly decreased over long follow-up time (Fig. 3).

Multivariate Analysis of Melanoma-Specific Survival

The presence of ciliary body involvement (HR, 2.32), LBD (HR, 1.15 for each unit increase in mm), presence of epithelioid cells (HR, 3.05), presence of microvascular loops and networks as modeled by assuming networks to be an advanced stage of loops (HR, 1.85 for each unit change in category), high MVD (HR, 1.37 for each unit increase in square root-transformed vessel count), and presence of heavy tumor pigmentation (HR, 2.94) were significantly associated with an increased risk of melanoma-related death, but the design variables modeling the predominant type of macrophages were not associated with survival. The number of macrophages did not fulfill the proportional hazards assumption ($\chi^2 = 5.48; 1$ df; $P = 0.019$, indicating that the risk changes over time, and hazard is not proportional) and was modeled by stratification.

A multivariate Cox regression model previously fitted to this data set was adjusted for the effect of immunopositive macrophages and tumor pigmentation. The presence of microvascular loops and networks, the presence of epithelioid cells, LBD, and MVD retained their significance in the model stratified by the number of CD68-immunopositive cells (Table 2).
staining patterns between mAbs against the CD68 epitope may result from variable glycosylation. Transient expression of the highly glycosylated and antigenically heterogeneous CD68 molecule than mAbs 3A5 and PG-M1.19,32

During differentiation of monocytes, this association has been taken as evidence that macrophages contain a number of cytokines and growth factors, which is another independent high-risk indicator for metastasis in uveal melanoma.4,10 However, immunopositive cells could cluster around these and other microvascular patterns, and a biologically significant qualitative association is not excluded by the present study.

An association between the number of infiltrating macrophages and high MVD has been recently observed in certain other cancers, including cutaneous melanoma.15-17 Because macrophages contain a number of cytokines and growth factors, this association has been taken as evidence that macrophages and presence of microvascular loops and networks, which is another independent high-risk indicator for metastasis in uveal melanoma.4,10 However, immunopositive cells could cluster around these and other microvascular patterns, and a biologically significant qualitative association is not excluded by the present study.

The number of macrophages was associated with three known high-risk indicators: LBD, presence of epithelioid cells, and high MVD in areas of densest vascularization.12,13 In spite of the different methodology of the COMS study, it also found significantly higher numbers of macrophages in uveal melanomas with epithelioid cells.30 Similarly, the number of infiltrating macrophages increased with LBD in both studies, and both showed a significant association between heavy pigmentation and a high number of infiltrating macrophages,40 suggesting that these associations are genuine. No association was observed between the number of macrophages and presence of microvascular loops and networks, which is another independent high-risk indicator for metastasis in uveal melanoma.4,10 However, immunopositive cells could cluster around these and other microvascular patterns, and a biologically significant qualitative association is not excluded by the present study.

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phages might promote angiogenesis. A statistical association is not proof of a causal relationship, however, and experimental studies are needed to investigate this theory. Especially uveal melanoma, in which the relative contribution of classic angiogenesis and tumor-cell-driven vasculogenesis is open, a more complex role for macrophages in remodeling microvasculature may apply.

Our population-based analysis of survival of 139 consecutive patients with choroidal and ciliary body melanoma showed a strong association between increased melanoma-specific mortality and increasing number of CD68-positive macrophages by univariate Kaplan-Meier analysis. The cumulative 10- and 20-year melanoma-specific probabilities of survival were 0.47 and 0.42 lower, respectively, when the number of infiltrating macrophages was high rather than low. No association between mortality and the predominant type of infiltrating macrophages was observed. When the number of infiltrating macrophages by stratification was considered, the fit of our Cox proportional hazards model improved significantly. Moreover, a clinically significant survival difference of 0.28 remained after adjusting for other factors.

It is important to determine which biological link, if any, exists between a high number of macrophages, presence of epithelioid cells, and high MVD, because this probably reveals useful insights into the progression and metastasis of uveal melanoma. The presence of a high number of macrophages in aggressive melanomas might either be an indication of a host response mounted against more malignant tumors, whether mediated by macrophages themselves or by other events, or it may simply be indirect evidence of an aggressive tumor that has a high cell turnover rate and, consequently, a greater demand for phagocytosing cells.

A corollary of the present study is that the presence of macrophages in uveal melanomas that have been conservatively managed cannot automatically be ascribed to treatment effect. Macrophages also constitute a nonneoplastic cell population that is a potential source for cross-reactivity in melanomas studied by immunohistochemical methods. The response of infiltrating macrophages to tumor destruction and their possible role in host defenses against metastasis after conservative management of uveal melanoma are relevant questions to be examined in future research.

References

30. Collaborative Ocular Melanoma Study Group. Histopathologic characteristics of uveal melanomas in eyes enucleated from the


