Microcirculation and Tumor-Infiltrating Macrophages in Choroidal and Ciliary Body Melanoma and Corresponding Metastases

Päivi Toivonen, Teemu Mäkitie, Emma Kujala, and Tero Kivelä

PURPOSE. To investigate the relationship between progression to hepatic metastasis and tumor-infiltrating macrophages and microcirculation attributes in uveal melanoma, a cancer that almost invariably disseminates hematogenously to the liver.

METHODS. A cross-sectional histopathologic analysis of 48 hepatic metastases and corresponding primary choroidal and ciliary body melanomas was conducted, by using statistical tests appropriate for paired data. Main outcome measures were the number and type of CD68-immunopositive, tumor-infiltrating macrophages, extravascular matrix loops and networks identified with periodic acid-Schiff stain, and microvascular density (MVD) counted as the number of discrete structures labeled by monoclonal antibody QBEND/10 to the CD34 epitope.

RESULTS. Hepatic metastases had a significantly lower grade of pigmentation \( (P < 0.0001) \), more frequent epithelioid cells \( (P = 0.0027) \), more intermediate and dendritic types of CD68-immunopositive macrophages than round ones \( (P = 0.0031) \), and a higher MVD \( (\text{median difference, } 15 \text{ counts more}/0.313 \text{immunopositive macrophages than round ones} \) than the primary uveal melanomas that spawned the metastases. The frequency of tumors with extravascular loops and networks did not increase on metastasizing. The survival of the patient after diagnosis of disseminated disease tended to be shorter if hepatic metastases had a high MVD \( (P = 0.098) \), adjusting for the size of the specimen.

CONCLUSIONS. Of the markers studied, the presence of epithelioid cells and MVD most closely parallel progression of uveal melanoma from primary tumor to metastasis. These two tumor characteristics may be interrelated, and high MVD may help to predict survival after detection of hepatic metastases. The results also suggest that the grade of pigmentation and morphologic type of tumor-infiltrating macrophages are interrelated. \( \text{(Invest Ophthalmol Vis Sci. 2004;45:1–6) DOI: 10.1167/iovs.03-0622} \)

From the Ocular Oncology Service, Department of Ophthalmology, Helsinki University Central Hospital, Helsinki, Finland.


Submitted for publication June 19, 2003; revised August 8 and August 27, 2003; accepted September 1, 2003.

Disclosure: P. Toivonen, None; T. Mäkitie, None; E. Kujala, None; T. Kivelä, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Paivi Toivonen, Department of Ophthalmology, Helsinki University Central Hospital, Haartmaninkatu 4 C, PL 220, FIN-00029 HUS, Helsinki, Finland; paivi.toivonen@hus.fi.

The evidence suggests that choroidal and ciliary body melanoma disseminates several years before diagnosis.\(^{1,2}\) It metastasizes almost invariably hematogenously and in up to 95% of the patients initially to the liver.\(^{3–6}\) The presence of epithelioid cells, extravascular matrix patterns that reflect the arrangement of tumor microcirculation,\(^{7–10}\) high microvascular density (MVD),\(^{11–13}\) and large numbers of tumor-infiltrating macrophages\(^{4,15}\) in primary uveal melanoma are independently associated with shorter time to metastatic death than the absence of these characteristics. These associations may be either markers of aggressive tumors without direct contribution to the metastatic cascade or they may indicate direct participation in tumor progression to metastasis.

Despite treatment with current chemotherapy regimens, patients with metastases from uveal melanoma usually die within 2 to 14 months after the diagnosis of disseminated disease by routine screening.\(^{16–18}\) The prognosis has improved only scantily over the decades, and part of the apparent improvement is likely to reflect lead-time bias from screening.\(^{17}\) A need exists to better understand the biology of and factors contributing to progression of disseminated uveal melanoma. One way to increase understanding of progression from primary to metastatic melanoma in humans is to compare how tumor characteristics change on dissemination. One study has compared extravascular matrix patterns in primary and metastatic choroidal and ciliary body melanomas, but it did not compare this attribute within patients.\(^{19}\) We undertook a paired analysis of primary choroidal and ciliary body melanomas and their corresponding hepatic metastases to test the hypothesis that microcirculation attributes and macrophage infiltration increase in grade with progression of primary tumor to metastasis. We also studied the association of microcirculatory attributes with survival after diagnosis of metastatic uveal melanoma.

PATIENTS AND METHODS

Eligibility Criteria and Enrollment

The study complied with the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board. All patients with a choroidal and ciliary body melanoma for which they had undergone enucleation of an eye in the district of the Helsinki University Central Hospital between 1962 and 1981 and later had metastasis were eligible. Enucleation was the standard treatment for all but the smallest melanomas during this period, making the series essentially population-based and unselected.

Inclusion criteria were that at least 50% of the primary tumors remained in the tissue block, and the remaining part was not entirely on the vitreal side of Bruch’s membrane,\(^{19}\) and that one or more core-needle biopsy, biopsy, or autopsy specimens with a surface area of at least 0.35 mm\(^2\) was available from hepatic metastases. This is roughly the minimum area needed to measure MVD.\(^{12}\)

During the study period, 292 consecutive patients had an eye with a choroidal and ciliary body melanoma removed, and 145 of these...
patients had metastases that were cytologically or histologically confirmed in 92 of them. Forty-eight pairs of primary tumors and hepatic metastases that fulfilled the inclusion criteria were identified (inclusion ratio, 33% of all patients with metastases: 29 women and 19 men).

The metastases had been recognized by liver imaging or laparoscopy after they caused symptoms. The original size of the biopsied and autopsied metastases was not recorded and, except in one case, the entire metastasis was not present in the specimen. The largest diameter in the biopsy specimen was measured from the sections with a pair of calipers. Of the 48 sections, 3 (6%) were obtained by core needle biopsy, 18 (38%) by surgical biopsy, and 27 (56%) at autopsy. The mean and ranges of specimen sizes for these three groups were 2 mm (range, 1.5–3), 7 mm (range, 2–18.5), and 20 mm (range, 6.5–35), respectively.

The disease-free interval was calculated as the time from enucleation to diagnosis of metastases, and overall survival as the time from the date of diagnosis of metastases to death. It was possible to calculate these intervals in the 22 patients who had undergone a biopsy rather than an autopsy.

**Immunoperoxidase Staining**

The paraffin blocks were cut at 5 μm. Immunostaining was performed using the avidin-biotinylated peroxidase complex method (Vectastain ABC Elite Kit; Mouse IgG, Vector Laboratories, Burlingame, CA), as described previously in detail.20 The mouse monoclonal antibody (mAb) HMB45 (diluted 1:100; lot 0024b; Dakopatts A/S, Glostrup, Denmark) to immature melanosomes, which labels more than 90% of uveal melanomas, was used to help to identify tumor cells from infiltrating macrophages,21,22 and mAb PG-M1 (diluted 1:50, IgG3; lot 101; Dakopatts) to the CD68 epitope was used to label macrophages. CD68 is an intracytoplasmic 110-kDa glycoprotein of lysosomal granules, which is expressed by macrophages in most human tissues. PG-M1 immunostains tumor-infiltrating macrophages in uveal melanoma more consistently than other tested anti-CD68 antibodies.13 Mouse mAb (QBEND/10, diluted 1:25; lot 121202; Novocastra Laboratories, Newcastle-upon-Tyne, UK) was used to label the CD34 epitope typical of endothelial cells.23 Pretreatment with 0.4% (wt/vol) pepsin (2500 FIPU; E. Merck, Darmstadt, Germany) in 0.01 M hydrochloric acid for 15 minutes at 37°C enhanced immunostaining and reduced background with all antibodies used. 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 overnight with 3.0% (vol/vol) hydrogen peroxide and 1.0% (wt/vol) disodium hydrogen phosphate, as described previously.24

**Histopathologic Data**

Outcome variables were assessed from non-necrotic areas. The grade of pigmentation (amelanotic to weak versus moderate versus strong) was determined by sorting unstained sections on white tissue paper under incandescent light.14 Cell type was registered as spindle if no epithelioid cells were found and nonspindle if one or more typical epithelioid cells were present. The number of mitotic figures per 10 high-power fields (HPFs; total area 2.6 mm²) was counted in hematoxylin–eosin-stained sections when the specimen was at least of this size.

**Grading of Infiltrating Macrophages**

The number of tumor-infiltrating macrophages was evaluated semi-quantitatively by comparing CD68-immunostained sections to published standard photographs (few versus moderate versus high numbers of cells).14 The predominant morphologic type of CD68-positive cells was likewise divided into three categories according to standard photographs: tumors in which the majority (75% or more) of immunopositive cells were either round or dendritic and tumors in which neither the dendritic nor the round type predominated or the morphology of immunopositive cells was intermediate.14 Confluent immunonegative cells in necrotic areas did not influence the grading. It was possible to evaluate all specimens but one metastasis. Analyses of tumor-infiltrating macrophages included all sizes of specimens, because these cells infiltrate uveal melanomas more or less diffusely.14

**Grading of Microcirculation Attributes**

Extravascular matrix loops and networks were assessed independently by two observers, according to the criteria of Folberg et al.7,25 Sections were bleached with 0.25% potassium permanganate and 5% oxalic acid and stained with periodic acid-Schiff without counterstain.7,25 Loops and networks were identified under a green filter (Wratten No 58; Eastman Kodak, Rochester, NY). By definition, networks consisted of at least three back-to-back loops.25 Discrepancies were resolved by consensus under a double-headed microscope. Specimens of hepatic metastases that were less than 4 mm19 were excluded from the analysis.

MVD was evaluated from the densest CD34-immunopositive area (hot spot) identified by scanning the entire CD34-immunostained tumor at ×100 magnification, according to the method of Foss et al.11,26 Immunopositive elements were counted at ×200 magnification, using an eyepiece with an etched square graticule corresponding to an area of 0.313 mm² (WK10x/20L-H; Olympus, Tokyo, Japan).12 Any immunolabeled element, clearly separate from adjacent ones and totally inside the graticule or touching its top or left border, was counted.7,12,20 Microcirculation attributes were assessable in all specimens but one metastasis.

**Statistical Analysis**

All analyses were performed with the Stata (release 7.0; Stata Co., College Station, TX) and StatXact-3 (Cytel Co., Cambridge, MA) statistical software packages. P < 0.05 was considered statistically significant, and all tests were two-tailed.

The median and range are given as descriptive statistics. The Wilcoxon signed-rank test was used to compare distributions of paired continuous data and the Stuart-Maxwell test and test for trend to compare unordered and ordered paired contingency tables, respectively.7 Spearman’s rank correlation was used to analyze interrelationships between two variables. The Mann-Whitney U-test was used to compare disease-free intervals between categories.

Overall survival was analyzed with the Kaplan-Meier product-limit method and log-rank test. MVD was divided into two groups according to the median. Cox regression was used to adjust for the size of the metastatic specimen.

**Results**

The median age of the 48 patients at enucleation was 55 years, and the median disease-free interval was 4.2 years (Table 1). For the 22 patients for whom the date of hepatic metastasis was known, the median time from metastasis to death was 1.9 months. The median height and largest basal diameter (LBD) of the primary tumor were 7 and 14 mm, respectively, and the median LBD of the specimen representing the metastasis was 16 mm (Table 1).

**Cell Type, Pigmentation, and Mitotic Count**

The hepatic metastasis contained epithelioid cells more often than did the primary tumor that had spawned it (P = 0.0027, Stuart-Maxwell test; Fig. 1A), and its grade of pigmentation was lower than that of the primary tumor (P < 0.0001, Stuart-Maxwell test for trend; Table 1). One primary tumor lost epithelioid cells on metastasizing, whereas 9 of 12 (75%; 95% CI, 43–95) spindle cell melanomas progressed to formation of epithelioid cells in hepatic metastases (Fig. 1A). The number of mitotic figures in specimens of hepatic metastasis was compa-
Microcirculation and Macrophages in Metastatic Melanoma

Tumor-Infiltrating Macrophages

The number of CD68-immunopositive cells in the matched primary tumors that metastasized was graded few in 4% and many in 46%. The number of CD68-positive cells in the corresponding hepatic metastasis was comparable to that in the primary tumor (P = 0.16; Stuart-Maxwell test for trend; Fig. 1C, Table 1).

The type of CD68-immunopositive cells in the matched primary tumors was predominantly round in 15%, intermediate in 71%, and dendritic in 15%. The distribution was shifted from round to dendritic in the metastases compared with the primary tumors, even though the intermediate type formed the majority in both groups (P = 0.0031; Stuart-Maxwell test; Fig. 1D, Table 1).

Extravascular Matrix Loops and Networks

Loops and networks were detected in 67% and 50%, respectively, of the 42 matched primary tumors from which a metastatic tissue sample at least 4 mm by LBD was available. These percentages were 74% and 60%, respectively, in hepatic metastases (Table 1), but no shift toward networks was observed on metastasizing compared with the primary tumor (P = 0.38 Stuart-Maxwell test for trend; Fig. 1E). Of 21 primary tumors with no loops or loops only, 12 (57%; 95% CI, 34–78) showed development of networks on metastasizing, whereas of 21 primary tumors with networks, 8 (38%; 95% CI, 18 to 62) no longer had networks identifiable in corresponding metastases (Fig. 1E). The presence of loops and networks did not correlate with the largest diameter of the specimen (p = −0.073, P = 0.65 Spearman’s rank correlation; Fig. 1F). Of the six specimens that were less than 4 mm in diameter, only one contained loops or networks.

Microvascular Density

Median MVD in the 47 matched primary melanomas was 41.5 counts/0.313 mm², significantly lower than 57 counts/0.313 mm² in the specimens from hepatic metastases (median difference, 15 counts more/0.313 mm² range, 38 counts less to 79 counts more; P = 0.0003 Wilcoxon signed rank test; Table 1). MVD in the hepatic metastases, as determined from the specimen available, was higher than that in the primary tumor in 31 patients (66%; 95% CI, 51–79; Fig. 1G).

MVD did not correlate with the largest diameter of the metastatic tissue sample (p = −0.064, P = 0.69 Spearman’s rank correlation; Fig. 1H). The range of MVD in specimens of metastases smaller than 4 mm was comparable to that among larger specimens (Fig. 1H).

Disease-Free Interval and Survival after Metastasis

The disease-free interval was associated neither with presence of extravascular matrix loops and networks (P = 0.50 Mann-Whitney U-test; Fig. 2A) nor with MVD in the hepatic metastases (P = 0.42; Fig. 2B).

The date of diagnosis of metastases was known for only two patients without loops in the metastasis, precluding a mean-

| Table 1. Comparison of Baseline and Outcome Characteristics between 48 Choroidal and Ciliary Body Melanomas and Their Corresponding Metastases |
|--------------------------|--------------------------|--------------------------|
| **Characteristic**       | **Primary Tumor**        | **Corresponding Liver Metastasis** |
| Male:female              | 19:29                    | N/A                      |
| Median age at enucleation, years (range) | 55 (23–77) | N/A                      |
| Median disease-free interval, years (range) | N/A                    | 4.2 (0.4–21.9)          |
| Median overall survival, months (range) | N/A                    | 1.9 (0.1–51.4)          |
| Ciliary body involvement, n (%) | Yes (15)                | N/A                      |
|                      | No (35)                  | N/A                      |
| Median height, mm (range) | 7.0 (2.0–16.0)          | N/A                      |
| Median LBD, mm (range) | 14 (6–21)               | N/A                      |
| Median size of metastatic specimen, mm (range) | N/A                    | 16 (1.5–55)             |
| Median mitotic count, per 10 HPF (range) | 1 (0–5)                | 1 (0–7)                 |
| Cell type, n (%) | Spindle (13)            | 3 (6)                    |
|                      | Nonspindle (35)         | 44 (94)                  |
| Grade of pigmentation, n (%) | Amelanotic (2)          | 26 (54)                  |
|                      | Moderate (25)           | 11 (25)                  |
|                      | Strong (21)             | 11 (25)                  |
| Number of infiltrating CD68-positive cells, n (%) | Few (2)                 | 0 (0)                    |
|                      | Moderate (24)           | 21 (45)                  |
|                      | High (22)               | 26 (55)                  |
| Type of infiltrating CD68-positive cells, n (%) | Round (7)               | 1 (2)                    |
|                      | Intermediate (34)       | 28 (60)                  |
|                      | Dendritic (7)           | 18 (38)                  |
| Microvascular pattern, n (%) | No loops (14)          | 11 (26)                  |
|                      | Loops without networks (7) | 6 (14)                  |
|                      | Networks (21)           | 25 (60)                  |
| Median MVD, counts/0.313 mm² (range) | 41.5 (11–96)          | 57 (19–128)             |

LBD, largest basal tumor diameter; HPF, high power field; MVD, microvascular density; N/A, not applicable.

* Excluding hepatic metastatic specimens less than 4 mm in largest diameter.
meaningful analysis of survival according to the presence or absence of loops and networks in the metastasis (Fig. 2C). Also, only two patients did not have epithelioid cells in the metastasis.

The overall survival tended to be shorter for patients whose hepatic metastases had an MVD higher than the median value compared with patients with a lower MVD (1.4 vs. 3.2 months, \( P = 0.11 \) log-rank test; Fig. 2D). A trend was observed when Cox regression was used to adjust for differences in size of the specimen available (hazards ratio, 1.02 for each unit increase in MVD, \( P = 0.098 \)).

**DISCUSSION**

Paired analysis of primarily enucleated choroidal and ciliary body melanomas and corresponding hepatic metastases re-
consequently, MVD in uveal melanoma might may express the CD34 epitope in contrast to nonaggressive aggressive melanoma cells, which are genetically deregulated, and death than low MVD.11 uveal melanoma is associated with a shorter time to metastasis analogously to the fact that a high MVD in primary metastases would also have a higher number of CD68-immunopositive cells than would primary uveal melanomas.

MVD is a histopathological indicator associated with tumor microvessels that has independent prognostic significance in uveal melanoma.11–13 Some investigators have suggested that aggressive melanoma cells, which are genetically deregulated, may express the CD34 epitope in contrast to nonaggressive tumor cells.13 Consequently, MVD in uveal melanoma might reflect tumor angiogenesis, aggressiveness, or both. In fact, high MVD in primary uveal melanoma is associated with presence of epithelioid cells,12 which also were more frequent in the metastases studied compared with the corresponding primary tumors. Analogous to the fact that a high MVD in primary uveal melanoma is associated with a shorter time to metastasis and death than low MVD,11–13 high MVD, as identified by CD34 expression, in hepatic metastasis tended to be associated with shorter survival after diagnosis of metastasis.

We did not have significant numbers of metastases from any other single site except liver. The possibility that MVD and CD34 expression might vary with tumor microenvironment remains to be studied. Six metastatic tissue specimens available were biopsy samples of less than 4 mm by LBD and may thus represent essentially random rather than hot-spot sampling to measure MVD, which in primary tumors blurs the association between MVD and prognosis.12,26 The observed difference between primary tumors and metastases could have been somewhat greater if larger samples of these six metastases had been available.

Disregarding the pairing of the specimens, the metastases had extravascular matrix networks more frequently than did the primary tumors. These observations are consistent with a previous report of primary and metastatic tumors from 19 patients.19 In our series, however, some metastases lacked loops and networks, whereas in the previous study all except one metastasis had networks and all had loops,19 two of several matrix patterns associated with death caused by metastasis.12–15 Because networks may be present only in foci within metastatic lesions, we excluded specimens of metastasis smaller than 4 mm from analysis of loops and networks.19 Despite this, in paired analysis, we did not find evidence that the frequency of networks would increase on progression to hepatic metastasis. Finally, primary tumors that metastasize have more frequent networks than primary uveal melanomas in general,7,9,10 making it more difficult to detect a further increase. Consequently, it is still premature to conclude that these matrix patterns would be unassociated with later progression of uveal melanoma.

Our paired analysis revealed that progression to hepatic metastasis was associated with preferential presence of the dendritic rather than round type of CD68-immunopositive cells, most likely tumor-infiltrating macrophages.14,30 within the tumor. This may at least in part reflect the observation that hepatic metastases were significantly less pigmented than the corresponding primary tumors. Weak pigmentation of primary uveal melanoma is strongly associated with the predominance of the dendritic type of infiltrating macrophages.14

In primary uveal melanoma, a high number of infiltrating macrophages is independently associated with shorter time to death resulting from metastasis.14 As mentioned, the present study was inconclusive as to whether the number of macrophages further increased on metastasizing. That metastases were mainly weakly to moderately pigmented probably made it difficult for us to prove an association between progression to metastasis and increase in tumor-infiltrating macrophages, because in primary tumors weak pigmentation is associated with a smaller than average number of infiltrating CD68-immunopositive cells.14

MVD potentially is modulated by the presence of epithelioid cells, some of which may express the CD34 epitope12 and, in primary tumors at least, by tumor-infiltrating macrophages.14 It would be worthwhile to develop an experimental model to study whether areas of high MVD are directly involved in escape of tumor cells from the primary and meta-
static tumors. The alternative hypothesis would be that high MVD merely re-ects the presence of aggressive tumor cells that are capable of progressive metastasis. Generalizing findings from animal models of uveal melanoma may be problematic, however, because the growth kinetics are different from that of humans, evidenced by the fact that experimental tumors can give rise to overt metastases within weeks. The alternative hypothesis would be that high MVD of biopsyed hepatic metastases may thus be evaluated as a supplementary means of estimating the prognosis of patients with metastatic uveal melanoma, in addition to the clinical parameters now available.

References
5. Gragoudas ES, Egan KM, Seddon JM, et al. Survival of patients with uveal melanoma to metastasis and death, in progression from the primary tumor to hepatic metastases and, as a new possibility, in survival after detection of hepatic metastases. MVD of biopsyed hepatic metastases may thus be evaluated as a supplementary means of estimating the prognosis of patients with metastatic uveal melanoma, in addition to the clinical parameters now available. 17