Background: Clinically undetectable or dormant metastases (micrometastases) probably account for disease recurrence, ie, clinically evident metastases, in patients after disease-free intervals of variable length. Recently developed animal models have shown that dormancy may potentially be explained by the fact that these micrometastases are not vascularized and have comparable rates of cellular proliferation and programmed cell death (apoptosis), enabling them to remain viable indefinitely but not to show progressive growth.

Observations: We report for the first time that melanoma micrometastases from humans are similarly not vascularized (mean number of microvessels, 10.2), have significantly lower rates of tumor cell proliferation (mean, 2.4%), and comparable rates of proliferation and apoptosis (means, 2.4% and 0.2%, respectively), compared with melanoma macrometastases, which have significantly greater tumor vascularity (mean number of microvessels, 18.7), higher rates of proliferation (mean, 18%), and higher rates of proliferation relative to apoptosis (means, 18% vs 1.6%). Tumor vascularity was quantified using the lectin *Ulex europaeus* agglutinin I to identify the number of microvessels per unit area (microscope ocular grid with an area of $7.84 \times 10^{-2}$ mm$^2$ at $\times 400$ magnification). Melanoma cell proliferation rate was assessed with the MIB-1 antibody (Ki-67) as the number of positive nuclei per total number of tumor nuclei counted at $\times 400$ magnification. Apoptosis was quantified using the method of terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate–biotin nick end labeling. The number of positive nuclei were quantified per total number of tumor nuclei; usually 200 tumor nuclei were counted at $\times 400$ magnification.

Conclusion: We report, for the first time, that human micrometastases demonstrate attributes, ie, the lack of significant tumor vascularity and low but comparable rates of proliferation and apoptosis, that may explain the dormant state.

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† Untitled Link

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MATERIALS AND METHODS

MELANOMA SPECIMENS

Eight microscopic (clinically undetectable) melanoma metastases were obtained from sentinel lymph nodes and 12 macroscopic (clinically palpable) melanoma lymph node metastases from patients at the following institutions: Brigham and Women’s Hospital, Boston, Mass; the University of Washington, Seattle; and the University of California at Los Angeles. The sentinel node procedure involves intraoperative mapping of the first lymph node encountered in the regional lymphatic drainage from a primary melanoma with a dye and/or imaging technique. The otherwise clinically undetectable lymph node is excised and examined for involvement by melanoma. Tumor vascularity, tumor proliferation rate, and rate of apoptosis were evaluated in tissue sections from formalin-fixed, paraffin-embedded material from the archives of the institutions listed above.

IMMUNOHISTOCHEMICAL DETECTION OF ULEX EUROPAEUS AGGLUTININ I AND THE MIB-1 ANTIBODY

Tumor vascularity was quantified as previously described using the lectin Ulex europaeus agglutinin I (UEA-I) to identify the number of microvessels per unit area (microscope ocular grid with an area of \(7.84 \times 10^{-2} \text{ mm}^2\) at \(400\) magnification). The single field judged to have the greatest number of microvessels was used to record the number of microvessels per unit area. Melanoma cell proliferation rate was assessed with the MIB-1 antibody (Ki-67) as the number of positive nuclei per total number of tumor nuclei counted at \(400\) magnification. In general, 200 tumor nuclei were counted, depending on the amount of tumor present for examination (range, 35 to 269 nuclei).

Four-micrometer-thick paraffin sections from human melanoma micrometastases and macrometastases were used for analysis after they were baked at 60°C for 30 minutes, deparaffinized, and rehydrated. For Ki-67 analysis, the tissue sections were then microwave treated (800 W, General Electric) at 199°F for 30 minutes in preheated 10-mmol/L citrate buffer at a pH of 6.0. For UEA-I analysis, the tissue sections were incubated with 0.1% trypsin solution at 37°C for 10 minutes. The slides were cooled for 15 minutes at room temperature (for Ki-67 analysis only), washed in phosphate-buffered saline, and then incubated with 2% horse serum for 15 minutes at room temperature. For Ki-67 analysis, the sections were incubated with Ki-67 antibody (Immunotech, Westbrook, Me) at a 1:100 dilution for 1 hour at room temperature, washed in phosphate-buffered saline, incubated with biotinylated horse antimouse IgG antibody (Vector Laboratories, Burlingame, Calif) for 30 minutes at room temperature, washed in phosphate-buffered saline, and then incubated with an ultrastreptavidin and biotinylated alkaline phosphatase complex (Signet Laboratories, Dedham, Mass) for 30 minutes at room temperature, followed by reaction with a red substrate kit (Vector Laboratories). For UEA-I analysis, the tissue sections were incubated with biotinylated UEA-I (Vector Laboratories) at a 1:80 dilution overnight at 4°C, washed in phosphate-buffered saline, incubated with ulcerase and biotinylated alkaline phosphatase complex for 30 minutes at room temperature, and subsequently treated as described above. All sections were subsequently stained with Mayer hematoxylin, cleared in xylene, and mounted.

QUANTIFICATION OF APOPTOSIS

Apoptosis was quantified using the method of terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate–biotin nick end labeling. Terminal deoxynucleotidyl transferase–labeling of formaldehyde-fixed tissue sections was performed according to Gavrieli et al, with the exception that sections were not pretreated with proteinase K before the terminal deoxynucleotidyl transferase–labeling reaction. Positive reactions were detected with a peroxidase-labeled antibody against deoxyuridine triphosphate–digoxigenin. The number of positive nuclei were quantified per total tumor nuclei; usually 200 tumor nuclei were counted at \(400\) magnification (range, 55 to 269 nuclei, depending on the amount of tumor present for examination).

The results were analyzed with the Wilcoxon rank sum test.

These studies constitute the first analysis of solid tumor micrometastases in humans. Before the development of the sentinel lymph node procedure, micrometastases were detected only by chance in surgical specimens, such as prophylactic lymphadenectomy specimens. However, with the advent of the sentinel node technique, there is now the singular opportunity to study the properties of a large number of micrometastases. In particular, these microscopic foci can be compared with clinically obvious (macroscopic) metastases, and whether they may in fact constitute dormant metastases in humans, as they have been described in animal preparations.

Our findings show that melanoma micrometastases are not vascularized to the degree of clinically de-
tectable macrometastases. The number of tumor microvessels in the micrometastases in general were not increased compared with the surrounding tissue, indicating that such metastatic foci have not switched to the angiogenic phenotype and thus are not yet capable of progressive growth. This scenario is directly analogous to that of primary tumors that have not achieved sufficient size and/or have not shown the transition to the angiogenic phenotype. The capacity for metastases to remain microscopic (and dormant) for long periods is hypothetical but is supported by clinical observations already mentioned; ie, the subsequent survival of patients after the development of metastases is fairly predictable irrespective of the length of antecedent disease-free intervals.

The micrometastases also exhibited much lower rates of tumor cell proliferation than the macrometastases. Although the proliferation rate was slightly greater than the rate of apoptosis, the rates are of a comparable order of magnitude. These results thus seem to indicate that microme-
tastases exhibit balanced rates of proliferation and apoptosis similar to those reported in experimental animal studies. These metastatic foci appear to have achieved a steady state in which tumor size remains small and constant and metabolic demands are not excessive because of relatively low rates of proliferation. Such a state can explain how micrometastases may remain dormant for many years or indefinitely. Because the rates of proliferation were slightly higher than the rates of apoptosis, one might argue that the 2 rates are in fact not balanced and that the micrometastases are growing. We cannot altogether rule out this possibility; however, we must emphasize that so few nuclei were recorded as positive with the 2 techniques that the differences in rates could probably be attributed to sampling or chance, and the differences are so small that they are in fact comparable. On the other hand, the macrometastases showed significantly greater rates of proliferation than apoptosis, consistent with progressive tumor growth and enlargement.

The concept of dormant metastases was hypothesized to explain why solid tumors, such as malignant melanoma and breast cancer, may develop metastases after disease intervals as long as 10 to 40 years after resection of the primary tumor and the failure of the wide surgical resection margins and elective lymph node dissection to clearly influence prognosis also supports the idea of occult metastasis in other anatomic locations.

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These findings suggest that as a group the micrometastases studied herein have not acquired the properties needed for progressive tumor growth, as evidenced by low tumor vascularity and proliferation rates. Our results suggest that these micrometastases may be analogous to dormant metastases, as described in animal models. However, we realize that there is likely to be heterogeneity among such a group of microscopic tumor deposits and that there is a need to study additional specimens. Nonetheless, this is the first study to analyze the properties of clinically undetectable metastases in humans and provides the first data on such a unique group of lesions.

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