Detection of Melanoma Cells in the Blood of Melanoma Patients by Melanoma-inhibitory Activity (MIA) Reverse Transcription-PCR

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Received 11/25/98; revised 2/1/99; accepted 2/3/99.

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The abbreviations used are: CAT, computer-assisted tomography; MIA, melanoma-inhibitory activity; RT, reverse transcription; PMBC, peripheral mononuclear blood cells; ED, evidence of disease; NED, no ED; DIG, digoxigenine; BIO, biotin; POD, peroxidase.

ABSTRACT

The detection of tumor-specific mRNA transcripts in the blood of patients by reverse transcription (RT)-PCR has been used as a very sensitive technique for determining systemically disseminated tumor cells. On the basis of previous expression studies, we aimed to trace melanoma cells in the blood of melanoma patients by RT-PCR of melanoma-inhibitory activity (MIA) mRNA. To detect sensitively MIA transcripts in total RNA isolated from peripheral blood mononuclear cells (PBMCs), we established a sensitive PCR-ELISA system. With this assay, we detected one melanoma cell in 2 ml of blood by a single round of 32 PCR cycles. A total of 295 PBMC samples isolated from 166 patients with melanocytic tumors were tested with the MIA RT-PCR-ELISA: (a) 58 patients (99 samples) with malignant melanomas in stage I; (b) 49 patients (65 samples) with malignant melanomas in stage II; and (c) 47 patients (116 samples) with metastasized melanomas (stages III and IV), with an additional 12 patients (15 samples) with benign melanocytic nevi. Forty-four (26.8%) of 164 samples isolated from patients with melanomas in stages I and II were positive for MIA mRNA; in stages III/IV, 33 (28.4%) of 116 samples of patients, irrespective of clinically evident disease, were positive. Eleven (84.6%) of 13 PBMC samples from patients with metastasized melanoma and clinically evident disease without treatment were MIA mRNA-positive in contrast to only 19 (25.7%) of 74 samples isolated from patients in stage IV with metastasis during chemotherapy. Furthermore, none of the 16 PBMC samples of patients in stage IV without clinically detectable metastases at that time point during chemotherapy was MIA mRNA-positive. Interestingly, of the 44 positive samples (26.8%) isolated from patients with melanomas in stages I and II, 20 were still positive when retested after complete excision of the tumor. Our results reveal that amplification of MIA mRNA from the PBMCs of patients with malignant melanomas by PCR-ELISA provides a useful means to detect tumor cells in the systemic blood circulation. A correlation between positive blood samples and tumor burden in stages III and IV was detected, and, in addition, a significant effect of chemotherapy with respect to the reduction of the number of systemically spread tumor cells was observed. However, MIA amplification seems to be of little value as a surrogate marker for clinical staging or the detection of metastatic disease.

INTRODUCTION

The incidence of malignant melanoma has been rising continuously during the past decade. Significant progress has been made in respect to detecting both the primary and the metastatic disease. After surgical removal of the primary tumor, patients are usually monitored during follow-up by clinical examination, routine laboratory tests, and in the case of suspicious findings by a number of imaging techniques including chest X-ray, and ultrasound, CAT, nuclear magnetic resonance, and postrion emission tomography scans. However, all of the currently available imaging techniques fail to detect micrometastases or tumor cells in the systemic blood circulation that are believed to precede clinically overt metastases. Presently, the most informative prognostic risk factors for systemic spread of melanomas in stage I or II are tumor thickness and regional lymph node status. The presence of melanoma cells in the systemic blood circulation has been suggested as an additional prognostic factor indicating an enhanced risk of metastasis (1–3). Other studies (3) have provided evidence that the detection of melanoma cells in the blood of patients in stages III and IV may reflect the extent of tumor burden or systemic tumor spread. We, therefore, aimed to extend currently available markers allowing for sensitive detection of tumor cells in the blood of melanoma patients by RT-PCR of cell type-specific gene transcripts.

Several lines of evidence suggest strongly that MIA may provide a novel molecular marker for detecting malignant melanoma cells. Previous reports from our own and other laboratories (4–7) have described very high levels of MIA expression in a wide variety of melanomas and melanoma cell lines. A correlation between MIA protein levels in the serum of patients with malignant melanomas and the progression to a systemic disease has been detected (8), and metastatic tumor cells express...
higher MIA levels than primary tumor cells. Most other cell types including keratinocytes, fibroblasts, melanocytes, and lymphocytes do not express MIA mRNA or else express extremely low levels—below the level of detection by a standard single-round RT-PCR (5). The only other tissue type known to express high levels of MIA mRNA is developing cartilage (9, 10).

Because of this restricted cell type-specific expression pattern resulting in very high mRNA levels in metastatic melanoma cells and encouraged by a previous preliminary report (11), we have addressed in this study whether MIA can be used as a sensitive and specific marker for disseminated melanoma cells in the blood. Previous studies (12) addressing this problem have amplified transcripts of prototypical genes of the mammalian pigmentary system including tyrosinase, TRP-1 and TRP-2. However, a series of inconsistent results obtained from performing tyrosinase RT-PCR have been published (12). Several groups (3, 13–16) have reported amplification of tyrosinase mRNA from the PBMCs of a high percentage of patients with metastasized malignant melanomas but not, or only infrequently, from healthy blood donors. However, others detected very few RT-PCR positive samples in melanoma patients or frequently false-positive samples in the control group (17–20).

To avoid at least some of the pitfalls of nested RT-PCR protocols including the very high risk of contamination, we established a PCR-ELISA system for MIA mRNA amplification based on a single-round RT-PCR.

MATERIALS AND METHODS

Melanoma Cell Lines. Melanoma cells were cultivated in DMEM supplemented with penicillin (400 units/ml), streptomycin (50 μg/ml), 1-glutamine (300 μg/ml), and 10% FCS under a humidified atmosphere of 8% CO2 at 37°C. Cells were detached for subcultivating or analysis with 0.1% trypsin and 0.04% EDTA in PBS and were used no later than 3 days after trypsinization. The melanoma cell line Mel Im was kindly provided by Dr. Johnson and Dr. Riethmüller (Institute of Immunology, University of Munich, Germany) and intensively characterized previously (21).

Patients, Isolation of PBMC, and Spiking of Blood Samples. Two hundred ninety-five blood samples from 154 patients with malignant melanomas (58 of stage I, 49 of stage II, 47 of stages III and IV) and from 12 patients with surgically removed benign melanocytic nevi were analyzed. Diagnosis was based on histological examination in all of the cases and, if necessary, was confirmed by S-100 and HMB 45 immunohistochemistry. Tumor stages, patients’ sex and age, and histological growth patterns are summarized in Table 1. Tumor thickness was determined according to Breslow’s criteria (22) and tumor stage according to the American Joint Committee on Cancer (23) as follows:

(a) stage I: T1–2, N0, M0;
(b) stage II: T3–4, N0, M0;
(c) stage III: T1–4, N1–2, M0; and
(d) stage IV: T1–4, N1–2, M1.

NED patients are defined as patients with no clinical ED based on physical examination and all of the imaging techniques as described below; ED patients are patients with clinically detectable metastases.

Serial blood samples from 16 patients during chemotherapy of stage IV melanoma were analyzed. Status of the patient was followed by clinical examination, routine laboratory tests, and tumor-size measurements by chest X-ray and ultrasound, CAT, and nuclear magnetic resonance scans.

A reference panel of 32 blood samples from 30 healthy blood donors (between 19 and 86 years of age (mean ± SD, 43.9 ± 19.2 years) was selected based on the following criteria: no use of medication, no record of any metabolic disorder, and no record of a malignant tumor (Table 1).

PBMCs were isolated from 3 ml of venous blood using the
sodium citrate containing Vacutainer CPT system (Becton Dickinson, Heidelberg, Germany). This technique is referred to as the Ficoll Hypaque method. It is based on a liquid density gradient of Ficoll 400 medium and sodium diatrizoate solution (24) and allows the isolation of the fraction of mononuclear cells. As described in the “Results” section, we verified the assumption that melanoma cells copurify with PBMCs.

To further control the sensitivity of the assay serial dilutions of the melanoma cell line Mel Im (1 to 10^6 cells) were performed in 2 ml of blood from healthy donors. Identical protocols for the isolation of PBMCs and RT-PCR ELISA were used in these blood-spiking experiments as for the blood specimens from melanoma patients.

**RNA Isolation and RT-PCR.** RNA was isolated from PBMCs with the RNasey kit (Qiagen, Hilden, Germany) following precisely the manufacturer’s instructions. One-fourth of the total cellular RNA was used as template for each RT-PCR amplification. RT-PCRs were performed in parallel for MIA (345 bp) and to control RNA quality for β-actin (600 bp) using the following primers: (a) MIA forward 5′-CAT GCA TGC GGT CCT ATG CCC AAG CTG-3′; (b) MIA reverse 5′-GAT AAG CTT TCA CTG GCA GTA GAA ATC-3′; (c) β-actin forward 5′-TGA CGG GGT CAC CCA CAC-3′; and (d) β-actin reverse 5′-CTA GAA GCA TTT GCG GTG GAA-3′. Primer pairs were designed across exon-intron borders to avoid amplification of genomic DNA. Primer for tyrosinase RT-PCR and reaction conditions were used as described previously (7). RT was performed in 20-μl reaction mix of 12 μl of RNA sample, 4 μl of 5× Superscript buffer (Life Technologies, Inc., Karlsruhe, Germany), 1 μl of 0.1 mm DTT, 1 μl of reverse primer (1 μg/μl), 1 μl of dNTPs (10 μM, DIG-labeled), and 1 μl of reverse transcriptase (Superscript, Life Technologies, Inc.). The mixture was incubated at 46°C for 45 min followed by 10 min at 70°C and RNase digestion at 37°C for 30 min. For PCR, 8 μl of 10× Taq buffer (Boehringer-Mannheim, Germany), 1 μl of forward primer (1 μg/μl), 1 μ l of dNTPs, 69.5 μl of H2O, and 0.5 μl of Taq-polymerase (Boehringer-Mannheim) were added to the reaction mix. Thirty-two cycles of PCR were performed using the following profile (5): 45 s at 94°C, 30 s at 55°C, and 60 s at 72°C. PCR reaction products were fractionated on 1.8% agarose gels. To control specificity, the PCR products were visualized on 1.8% agarose gels and confirmed by sequencing.

**PCR-ELISA.** To enhance sensitivity, a PCR-ELISA system (Boehringer-Mannheim) for detection of MIA and β-actin PCR products was used. Briefly, 20 μl of the PCR product was denatured; hybridized to a 5′-DIG-labeled, MIA- or β-actin-specific probe (BIO-MIA1: 5′-GAC TGC CGA TTC CTG AC-3′; BIO-MIA2: 5′-AAT CTC CCT GAA CGC TG-3′, BIO-β-actin: 5′-ACA CTG TGC CCA TCT ACG AG-3′); and bound to streptavidin-coated 96-well plates. Two different MIA-specific probes were used in parallel, and results were identical for all of the reactions. Finally, the immobilized PCR product was detected with an anti-DIG-POD antibody, visualized by a color reaction using the substrate tetramethylbenzidine and semiquantified photometrically. A schematic illustration of the ELISA is depicted in Fig. 1. Reactions more than 1.5-fold of the negative control were counted as positive. Negative control reactions with PBMCs of healthy blood donors were assayed in parallel to every ELISA.

**RESULTS**

**Sensitivity of Melanoma Cell Detection in PBMC by RT-PCR ELISA.** Calibrating the ELISA, we could measure MIA PCR products at least 20-fold more sensitively than by gel electrophoresis. When 10 μl of 40 μl of extracted total RNA were used as PCR template, we easily detected 0.5 melanoma cells in 1 ml of spiked blood samples by PCR-ELISA but only a minimum of 10 cells on ethidium bromide-stained agarose gels (Fig. 2, A and B). Detection of 1 cell in 2 ml of blood is within the sensitivity reported by other groups using nested PCR protocols (3, 15, 17, 25). To control PCR performance and the quality of template RNA and to compare the quantity of PCR products, β-actin RT-PCR was performed in parallel and was also measured by PCR-ELISA. In all of the cases, analysis by gel electrophoresis and ELISA was performed in parallel.

Peripheral blood cells were isolated using the Vacutainer CPT system (see “Materials and Methods”), which separates PBMCs from granulocytes, erythrocytes and plasma. RNA of PBMCs was isolated and tested for MIA expression. Consistent with previous results by Jung et al., (26) PCR amplification of RNA isolated from PBMCs was compared with the isolation of RNA from whole blood and was found to be more sensitive and reliable (data not shown).

MIA mRNA was detected in none of 32 PBMC specimens from 30 healthy blood donors, in contrast to 3 positive amplifications of tyrosinase mRNA in healthy donors (data not shown). Thirteen of 15 samples collected from 12 patients preoperatively who underwent surgery for clinically suspected melanoma but turned out to have benign melanocytic nevi after histological examination were negative. The two positive samples were obtained preoperatively from patients with a congenital nevus and a spindle cell nevus, respectively.
Detection of Circulating Melanoma Cells in Stages I and II.

Altogether, 280 PBMC samples collected from 154 patients with malignant melanomas were tested for MIA mRNA both by PCR-ELISA and by agarose gel electrophoresis (Table 2). In only 15 samples, the PCR product of MIA RT-PCR was detectable on ethidium bromide-stained agarose gels. All of these 15 samples were also strongly positive when analyzed in parallel by RT-PCR ELISA. Of the 280 samples, 99 samples were from 58 patients in stage I, and an additional 65 samples were from 49 patients in stage II. Forty-four (26.8%) of the 164 samples of patients with stages I and II melanoma tested positive. Before surgical removal of the tumor, we detected 24 (32.4%) MIA-positive samples in the 74 samples tested. Within 6 months of follow-up, five of these MIA-positive patients developed regional lymph node metastases. Interestingly, an additional 20 (22.2%) samples of 90 patients after surgery tested positive; 8 patients testing positive had been MIA-negative preoperatively. None of the patients who tested positive after surgery but negative before surgery progressed to metastatic disease within 3 months to 1 year of follow-up. In total, there were 40 patients tested pre- and postoperatively as a direct comparison. In 6 (15%) patients, we observed a change from MIA-positive to MIA-negative blood samples. Three patients (7.5%) stayed MIA-positive even after surgery. Thirty-one patients were MIA-negative before surgery of which 23 (57.5%) stayed negative and 8 (20%) became positive.

Comparing the growth pattern of the tumor and the result of the MIA RT-PCR analysis, we failed to detect any significant correlation.

Detection of Systemically Disseminated Melanoma Cells in Stages III/IV.

In 11 (84.6%) of 13 samples collected from patients with clinically evident metastatic disease and without current treatment, melanoma cells could be traced in the blood by MIA RT-PCR (Table 2). Furthermore, 74 samples from patients with clinically evident metastasized melanomas were tested during treatment with combined immunochemotherapy. Fifty-five (74.3%) of the samples tested negative, whereas MIA-positive cells were found in 19 samples (25.7%). There was no obvious difference in response to therapy between the two groups.

Testing patients in stage III/IV without clinically detectable metastases at the time point of evaluation, we did not detect MIA mRNA in any of 16 samples collected during treatment, but we did in 3 specimens (from 3 different patients) of 13 samples collected from patients without therapy (Table 2). Two

Table 2 Detection of MIA mRNA in samples from melanoma patients

<table>
<thead>
<tr>
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<th>Positive</th>
<th>Negative</th>
<th>Total</th>
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<tr>
<td>All</td>
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<td>72</td>
<td>99</td>
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<tr>
<td>Prior exc.</td>
<td>14</td>
<td>39</td>
<td>53</td>
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<tr>
<td>After exc.</td>
<td>13</td>
<td>33</td>
<td>46</td>
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<tr>
<td>Stage II</td>
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<tr>
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<td>48</td>
<td>65</td>
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<tr>
<td>Prior exc.</td>
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<td>11</td>
<td>21</td>
</tr>
<tr>
<td>After exc.</td>
<td>7</td>
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<td>44</td>
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<tr>
<td>Stage III/IV</td>
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Fig. 2 Comparison of the detection limits of MIA PCR products in spiked blood samples by agarose gel electrophoresis (A) or PCR-ELISA (B). A, gel electrophoresis (1.8% agarose gel) of the PCR products using 10 μl of 40 μl of PBMC RNA as template. The number of melanoma cells per 1 ml of blood is indicated. B, analysis of the same PCR products quantified by the ELISA. White columns, PCR reactions using 1 μl of template RNA; black columns, PCR reactions using 10 μl of template RNA. The number of melanoma cells per 1 ml of blood is indicated.
of these three MIA-positive NED-patients relapsed rapidly to clinically overt metastatic disease within the follow-up period of 6 months.

**Evaluation of Follow-Up Measurements in Stage III/IV as a Prognostic Tool.** Sixteen patients were available for serial testing at various time points during follow-up (summarized in Table 3). Variable positive and negative MIA RT-PCR results were obtained from PBMCs of 9 of the 16 patients (P7, P18, P36, P46, P58, P92, P96, P105, and P139) without any obvious correlation to the course of the disease. In three patients (P12, P14, and P54) the blood turned MIA-positive in parallel to an increase in the tumor burden during disease progression. Three patients (P17, P23, and P85) stayed always negative over the entire period of testing despite a large clinically evident tumor burden and widespread metastases. And one patient (P29) tested MIA-positive before surgery and turned MIA-negative after the removal of metastases (Table 3). In summary, these data illustrate that the course of disease and the results of MIA RT-PCR did not correlate in the majority of our patients.

**DISCUSSION**

Previous efforts to monitor the systemic spread of tumor cells in patients with malignant melanoma have been based on detection of tyrosinase mRNA-positive cells in the peripheral blood (3, 12, 15, 17, 20, 25, 27, 28). However, tyrosinase expression occurs in a number of different cell types including benign melanocytes and is lost in some malignant melanomas. Because of its highly restricted expression pattern, MIA mRNA has been proposed as an ideal surrogate marker for detecting systemically disseminated melanoma cells (11).

In this study, we have, therefore, established a nonradioactive, highly sensitive test that allows the detection of melanoma cells in the blood by RT-PCR of MIA mRNA. Our data show that the sensitivity of the PCR-ELISA system, one spiked melanoma cell in 2 ml of peripheral blood is equivalent to radioactive Southern blot analysis of single-round PCR protocols and to nonradioactive analysis of two-step nested PCR amplification. Tests based on nested PCR reactions are prone to cross-contamination and require extreme care and logistic effort when performed as routine large-scale analyses.

Previous studies have reported a variable number of positive tyrosinase amplifications from the PBMCs of healthy blood donors. Contamination by skin melanocytes during blood sampling has been suggested as a possible source for tyrosinase transcripts (16). This contamination does not appear to impose a problem for MIA RT-PCR because, in contrast to tyrosinase, MIA is not expressed at significant levels in benign melanocytes (5). Consistently, we found 3 tyrosinase RT-PCR-positive samples among 32 samples collected from healthy blood donors, whereas MIA RT-PCR amplification was negative in all of the 32 cases (data not shown). Together with the finding that tyrosinase expression is lost in a certain percentage of advanced or amelanotic melanomas (29, 30), it, therefore, seems that MIA provides a more reliable RT-PCR marker for sensitive and specific detection of disseminated melanoma cells in the blood circulation than tyrosinase.

**Detection of Disseminated Melanoma Cells in Early Stages of Disease.** The vast majority (73.1%) of blood samples from patients in stages I and II tested negative for MIA mRNA, which indicates that systemic tumor cell dissemination occurs only in a specific subset of primary melanomas. Twenty-four (14.6%) of the positive samples were collected before surgical removal of the tumor, but 20 samples collected within 24 h after surgery also tested positive.

Interestingly, an unusually high percentage (5 of 24) of patients with positive RT-PCR tests before surgery progressed...
to systemic disease within the follow-up period of 6 months. Because of the low number of samples, we are unable to draw any definite conclusion at this point of our study, but these data clearly require further investigation with respect to the possibility that a positive MIA RT-PCR test obtained before surgery may provide an independent risk factor for disease progression of early stage melanomas.

In contrast, none of the 20 patients with MIA-positive samples collected postoperatively had developed metastases within the follow-up period. It can be speculated that surgical removal of the primary melanoma causes significant cell shedding into the systemic blood circulation, which has been demonstrated for many other tumor types (31). However, based on our data, this phenomenon does not appear to be paralleled by a significant increase in tumor progression. Because we never observed positive test results in the 32 samples of healthy blood donors, we do not believe that positive MIA PCR amplification obtained postoperatively results from contamination with other cell types in the blood that express MIA mRNA.

Detection of Disseminated Melanoma Cells in Advanced Stages of Disease. In the group of patients in stages III and IV with clinically evident metastases but without any concurrent adjuvant therapy (ED patients), we measured 84.6% MIA-positive blood samples. Equivalent data have been obtained by a number of groups evaluating the sensitivity of tyrosinase RT-PCR amplification (3, 13), whereas other groups have reported a significantly lower percentage (17, 19, 20, 25). In stage III or IV patients that had no clinically evident metastases at the time of blood sampling (NED patients), we failed to detect any positive sample in the group of patients with concurrent adjuvant therapy, and only 23% of the samples (from 3 patients) of patients without adjuvant therapy tested positive. Although, MIA RT-PCR significantly discriminated between ED and NED patients, we observed many MIA-negative test results in ED patients and, therefore, did not obtain any staging information in addition to the information based on clinical and imaging data. However, two of the three NED patients with positive MIA RT-PCR results developed rapidly recurrent metastases pointing to the possibility that the detection of MIA in PBMCs of NED patients indicates imminent tumor relapse.

In addition, we observed a significant effect of adjuvant therapies on the number of positive blood samples in stage III/IV. In the group of ED patients, the percentage of positive samples dropped from 84.6 to 25.7%; in the group of NED patients, it dropped from 23 to 0%. This observation was also reported previously by Brossart et al. (32) based on tyrosinase amplification. However, others have failed to confirm a significant effect of therapy on the detection of melanoma cells in the blood (33). Although in our study, therapy resulted in a significant reduction of MIA-positive cells in the peripheral blood, we did not see an obvious difference in response to therapy.

We recently published (8) an MIA-ELISA system to measure MIA protein in the serum of melanoma patients as a prognostic marker in tumor follow-up and therapy monitoring. In contrast to detecting melanoma cells in the systemic blood circulation, MIA protein detection parallels the development of overall tumor burden. Therefore, these two assays assess different parameters of tumor biology and must not be compared.

In summary, significant differences of MIA RT-PCR results were observed between ED and NED patients and between patients in adjuvant immunochemotherapy and without therapy. However, test results did not correlate well with the course of the disease and did not provide additional staging information. Taken together, our data support the recent doubts concerning the use of RT-PCR-based detection of melanoma cells in the blood (17). Therefore, with the exception of preoperative analyses in stage I or II patients and screening NED patients in stage III/IV without therapy, we currently cannot recommend the test as a routine diagnostic tool. Whether detection of MIA-positive cells in the peripheral blood before surgical removal of early stage melanomas provides an additional risk parameter of disease progression requires additional investigation.

ACKNOWLEDGMENTS

We thank Ute Holzapfel for technical assistance and Michaela Golob and Max Kroiss for helpful discussions.

REFERENCES

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