Journal of Clinical Laboratory Analysis 13:229–233 (1999)

Detection of Circulating Melanoma Cells by Immunomagnetic Cell Sorting

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We developed a cellular approach to the identification of circulating melanoma cells in peripheral blood using immunomagnetic cell sorting. One hundred seventy-eight blood samples from 129 melanoma patients and 30 samples from healthy persons and nonmelanoma patients were examined. After density gradient centrifugation the interphase was incubated with the mAb 9.2.27. Positive cells were labeled with magnetic microbeads and enriched by immunomagnetic cell sorting. Cells were stained using an alkaline phosphatase–antialkaline phosphatase assay and examined by light microscopy. In spiking experiments, melanoma cells seeded at a concentration of one melanoma cell per ml whole blood could be detected reliably with the assay. Circulating melanoma cells were not found in 30 controls examined, nor were 9.2.27-positive cells found in 41 patients with primary malignant melanoma. In patients with regional lymph node metastases and in patients with disseminated disease, circulating 9.2.27-positive cells could be detected in 3 out of 22 patients (13.6%) and 10 out of 66 patients (15.2%) examined. We present a sensitive and specific immunocytological approach to detect circulating melanoma cells in peripheral blood. The method is not suitable for early detection of metastases but is a valuable tool for further investigating biological characteristics of circulating melanoma cells. J. Clin. Lab. Anal. 13:229–233, 1999.

Key words: neoplasm-circulating-cells; melanoma-blood; immunomagnetic-separation; cell-separation-methods; immunohistochemistry; skin-neoplasms-blood

INTRODUCTION

The incidence of malignant melanoma has increased during the last decade at a rate faster than any other cancer. The most alarming aspect of malignant melanoma is its potential to metastasize at a very early stage of the disease. In most instances the route of metastasis to distant organs is through the bloodstream (1).

Detection of tumor cells in the bloodstream has been the object of numerous investigations in various solid tumors. The methods used are either based on cellular examination techniques or on molecular biological methods. Cellular methods such as morphology, flow cytometry, and conventional cytogenetics have been shown to detect circulating tumor cells in solid tumors at levels of 1 in 100 mononuclear cells, and the more sensitive method of immunocytochemistry may detect one tumor cell in $10^5$to 10^6 mononuclear cells (2–6). Molecular biological methods are recently reported to detect one tumor cell in 10^7 mononuclear cells (7). Detection of tumor cells in peripheral blood by immunocytochemical assays has been reported for breast cancer (5,8–11), colorectal cancer (12,13), prostate cancer (14), and neuroblastoma (4). In malignant melanoma recent investigations have concentrated on molecular biological methods for detecting circulating melanoma cells (15–27). The assay most often used is based on the detection of tyrosinase messenger RNA expression by reverse-transcription polymerase chain reaction (RT-PCR). The method is considered to be extremely sensitive. However, the detection rates vary considerably. In patients with distant metastases, previously published detection levels vary from 0 to 100% (23–26). Most recent reports state that polymerase chain reaction-based detection of circulating melanoma cells is not suitable for early detection of metastasis (21,22). However, other investigators found that it might be an effective marker of tumor progression (17,19,27).

We developed a cellular approach to detect circulating melanoma cells using an immunocytologic assay with tumor

Grant sponsor: Federal Ministry of Education, Science, Research and Technology; Grant number: 01KS9602; Grant sponsor: Interdisciplinary Clinical Research Center (IKFZ), Tübingen.

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Received 25 March 1999; Accepted 14 April 1999

cell enrichment by magnetic activated cell sorting. As a marker for melanoma cells the murine monoclonal antibody 9.2.27, which is directed against a human melanoma-associated chondroitin sulfate proteoglycan (MCSP), was used (28). Tumor cells (9.2.27-positive) were enriched by magnetic activated cell sorting (MACS) and examined by light microscopy after immunocytochemical staining.

This objectives of this study were twofold: (1) to determine by spiking experiments whether single melanoma cells suspended in whole blood can be detected reliably with this assay; and (2) to assess whether circulating tumor cells in peripheral blood of melanoma patients may be identified and whether there is any association with clinical stage of disease.

PATIENTS AND METHODS

Melanoma Cell Line

The human melanoma cell line SK-Mel-30 (German Collection of Microorganisms and Cell Culture, DSM, Brunswick, Germany) was used for recovery experiments and as a positive control.

Antibodies

The murine mAb 9.2.27 is of IgG2a isotype and directed against a M_r 250.000 human melanoma-associated chondroitin sulfate proteoglycan (MCSP)(28). Purified, lyophilized antibody was kindly provided by Prof. Dr. R. A. Reisfeld (Department of Immunology, The Scripps Rerearch Institute, La Jolla, CA) and used at a final concentration of 5μg/ml. For magnetic labelling, goat antimouse IgG microBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were used.

Patients

The study included 129 consecutively selected melanoma patients (67 males, 62 females, mean age 51 years, range 17–84 years). Forty-one patients presented with primary cutaneous melanoma (American Joint Committee on Cancer Staging stages I and II), 22 patients had lymph node and/or in transit metastases (AJCC stage III), and 66 patients presented with distant metastases (AJCC stage IV) at the time the blood sample was taken. An additional 30 healthy individuals and nonmelanoma patients were examined. Two or more blood samples were analysed in 28 patients at different times resulting in a total of 208 blood samples being examined. Blood was taken from 10 patients with clinical stage I and II disease before surgical removal of the primary tumor wheras in 31 patients blood samples were collected after complete removal of the tumor. In patients with stage III melanoma, blood samples were taken before surgical removal of metastases in 13 cases and after removal of metastases in 15 cases. All patients were treated and followed-up at the Department of Dermatology, Universitäts-Hautklinik Tübingen, Germany, from March, 1997, until November, 1998.

Blood Collection and Separation of Erythrocytes

Peripheral venous blood (50 ml) was collected into a syringe containing 200 IU Heparin Natrium (Vetren® 200, Byk Gulden, Konstanz, Germany) and diluted with PBS Special® (Gibco BRL, Eggenstein, Germany). Leukocytes and tumor cells were separated from erythrocytes by layering specimens over Lymphoprep® density-gradient medium (Lymphoprep Density 1.077 g/ml, Nycomed, Oslo, Norway), followed by centrifugation at 800*g*, 20°C for 15 min. The whole fraction above 1.077 g/ml was harvested, concentrated by centrifugation and washed twice with PBS supplemented with 2mM EDTA.

Magnetic Cell Separation

The leucocyte-tumour cell fraction was resuspended in 400 μl PBS, supplemented with 2mM EDTA and incubated with 100 μl Polyglobin N® (Bayer, Leverkusen, Germany) for 10 min at 4°C. The monoclonal antibody 9.2.27 was added at a final concentration of 5 μg/ml and incubated at 4°C for 30 min. Cells were washed twice, resuspended in 400 μl PBS supplemented with 2mM EDTA, incubated with 100 μl Goat-Anti Mouse MicroBeads at 8°C for 20 min, and washed twice with PBS supplemented with 2mM EDTA and 0.5% BSA. The cell suspension was passed over a LS+ separation column (Miltenyi Biotec, Bergisch Gladbach, Germany) placed in the magnetic field of a Midi MACS separator (Miltenyi Biotec). Labelled cells attach to the magnetisized matrix in the column; unlabelled cell were removed by washing out with PBS supplemented with 2mM EDTA and 0.5% BSA, and collected as negative fraction. The separation column was removed from the magnet and the retained cells were flushed from the column with 5ml PBS supplemented with 2mM EDTA and 0.5% BSA and collected as positive fraction. Cells of this positive fraction were concentrated by centrifugation and attached to poly-l-lysine-covered glass slides (Sigma, Deisenhofen, Germany) which were then air dried and stored at 8°C for at most 24 hr.

Staining of Cells

Cells were fixed in acetone and chloroforme. Pure culture preparations of the SK-Mel-30 cell line were used as positive controls and incubated with the mAb 9.2.27. Because the positive and negative fractions of the blood samples had already been incubated with mAb 9.2.27 before magnetic activated cell sorting, no additional primary antibody was added to these samples. Preparations of SK-Mel 30 cells without incubation with 9.2.27 served as negative controls.

The antibody reaction was developed with the indirect immunoenzyme APAAP (alkaline phosphatase–antialkaline phosphatase technique, Dako, Hamburg, Germany) and cells were counterstained with Mayer's Hämalaun (Merck, Darmstadt, Germany). Red staining of positive cells was assessed by light microscopy.

Recovery Experiments

Decreasing numbers of SK-Mel-30 (1000, 500, 250, 50) were added to 50 ml blood of healthy volunteers. Another blood sample with no added tumor cells served as a control. The experiments were done under identical conditions as described above and repeated five times.

The viability of recovered melanoma cells was determined by reculture of eluted labeled cells in RPMI medium containing 10% FCS.

Evaluation Criteria

Melanoma cells were identified both immunologically by positive immunostaining detected on at least 50% of the surface membrane and morphologically. In all cases the entire positive fraction of a blood sample was examined without knowledge of the clinical stage of the patient. To minimize the possibility of a false-positive analysis, detection of at least two positive cells was required before a blood specimen was recorded as positive.

RESULTS

Sensitivity

In spiking experiments, melanoma cells seeded at a concentration of one melanoma cell per ml whole blood could be detected reliably in five independent experiments (Fig. 1). This corresponds to a sensitivity of one melanoma cell in more than 1×10^6 mononuclear cells. No positive cells were detectable in control samples. The recovery rate was consistently more than 10% of tumor cells seeded in the blood samples (mean 11.4%). The recovered cells could be easily regrown under standard culture conditions.

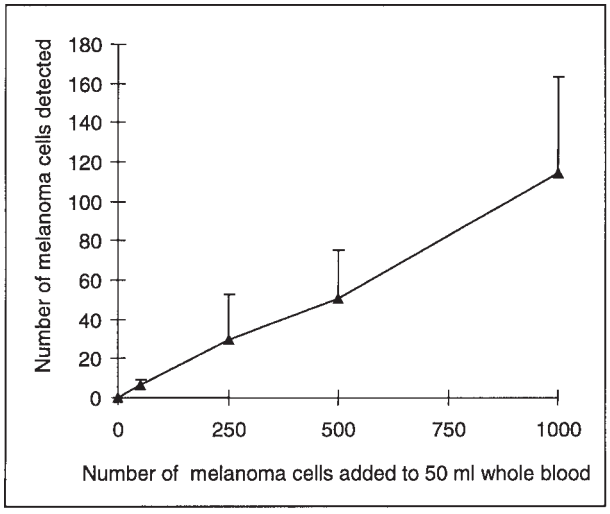

Fig. 1. In spiking experiments melanoma cells seeded at a concentration of one melanoma cell per ml whole blood could be detected reliably with the assay. The mean recovery was 11.4%.

Specificity

No positive staining was detected in the specimens from 30 healthy donors and nonmelanoma patients. All positive cells of melanoma patients revealed morphological criteria of tumor cells such as abnormal size and a high nuclear/cytoplasmatic ratio. Positive staining of WBCs was not seen in melanoma patients.

Detection of Circulating Melanoma Cells

One hundred seventy-eight blood samples from 129 melanoma patients and 30 samples from healthy subjects or nonmelanoma patients were tested for circulating cells in peripheral blood. In 28 patients two or more blood samples were analyzed at different times.

Clinical stage I and II (AJCC)

Circulating melanoma cells could not be detected in either 10 patients before surgical excision of primary tumor or in 31 patients after surgical removal of the primary tumor.

Clinical stage III (AJCC)

Thirteen blood samples were collected before surgery and 15 blood samples were examined after complete removal of metastases. In 3 of 22 patients examined (13.6%), 9.2.27-positive cells were found. All three patients had clinically detectable metastases at the time of analysis. A second blood sample was taken from one of these patients after complete surgical removal of all metastases and was found to be negative. The number of positive cells detected per sample ranged from 2 to 8. All positive cells revealed morphologic criteria of malignant cells.

Clinical stage IV (AJCC)

All patients examined presented with clinical evidence of metastases at the time of analysis. Circulating cells were detecetd in 10 out of 66 patients (15.2%) examined. The number of positive cells detected per blood sample ranged from two to 2,364 positive cells. All positive cells showed typical features of malignant cells.

DISCUSSION

The accepted model of metastasis includes shedding and intravascular transport of tumor cells. However, observation of this process is difficult and our understanding of the intravascular stage of metastasis is rather limited. The relevance of rate and timing of cell shedding for clinical outcome remains uncertain.

Recent investigations of malignant melanoma focused on molecular biological examination techniques for the detection of circulating melanoma cells using RT-PCR for detection of tyrosinase mRNA (15–27). One of the main

disadvantages of PCR is that it is an indirect method without a morphological correlate and cannot provide direct evidence for the actual presence of intact cancer cells in the blood containing these expressed mRNA sequences.

To overcome this limitation we developed a new method for detecting circulating melanoma cells based on a cellular examination technique using an immunocytologic assay with immunomagnetic cell enrichment. Detection of circulating cells by immunocytochemistry can be limited by two major problems. First, the technique is dependent upon the availability of antibodies to tumor-associated cell-surface antigens and may be subject to false positives when antibodies crossreact with mononuclear cells or tumor antigens that are expressed on host immune cells (29). As a marker for melanoma cells we chose the murine monoclonal antibody 9.2.27 which recognizes the core protein (250 kD) of a human melanoma-associated chondroitin sulfate proteogycan (MCSP) (28). The antigen is uniformly expressed on > 90% of human melanoma tissues and cultured cells (30,31). Mab 9.2.27 selectively targets MCSP on the melanoma cell surface after intravenous administration (32). Importantly, we demonstrated by FACS analysis that mononuclear cells do not express this antigen (data not shown). Second, immunocytochemistry may be limited by the fact that the procedure is both tedious and time consuming. Because the sensitivity of the method is directly dependent on the amount of blood examined, a large number of slides have to be examined at a low tumor cell/mononuclear cell ratio. Some means of enrichment is therefore essential. In our assay using a blood sample of 50 ml, tumor cells were enriched by magnetic activated cell sorting (MACS). Because the majority of mononuclear cells is eliminated by magnetic activated cell sorting, only a few slides have to be analyzed after immunocytochemical staining.

We first asked the question whether the assay is suitable for specific and sensitive detection of single melanoma cells suspended in whole blood. In this regard, no false positive cells were found in the blood of 30 healthy persons and nonmelanoma patients examined. In spiking experiments we could demonstrate that one melanoma cell per ml whole blood can be reliably detected with this assay. The sensitivity reported for detection of tyrosinase mRNA in model experiments is in the same range (19,22,23) or slightly higher (20).

Our second aim was to determine whether circulating melanoma cells may be identified in melanoma patients and whether there is an association with clinical stage of disease. To our knowledge, this is the first report to demonstrate that 9.2.27-positive melanoma cells with typical morphological characteristics of malignant cells may be identified in a number of melanoma patients with advanced disease. Thus 9.2.27-positive cells were found in 3 of 22 patients with stage III melanoma and in 10 of 66 patients with disseminated disease. All patients that reacted positively had clinically detectable metastases, some with huge tumor masses at the time of examination. The number of positive cells detected per blood sample varied widely. In most patients, two to eight positive cells were recorded. Assuming a mean recovery rate of 11%, found in our spiking experiments, this corresponds to 0.4 to 1.4 melanoma cells per ml whole blood. In three patients, who presented with fulminant progression of all known metastases, between 44 and 2,364 positive cells were detected. This corresponds to 8 to 426 melanoma cells per ml whole blood. Positive cells were not detected in patients with primary tumor, independent of whether the blood sample was taken before or after surgery, nor in patients after surgical removal of regional lymph nodes and/or in transit metastases.

Given the relatively low percentage of positive cells in the circulation even in patients with advanced disease seen in our study, a negative result certainly does not exclude the existence of metastases or micrometastatic disease. Consequently this assay cannot be used as a diagnostic procedure for melanoma staging. However, our preliminary results suggest that a positive result could be indicative of a high risk of relapse or of ongoing progressive disease. All three patients with clinical stage III disease that tested positively progressed four to eight weeks after surgical removal of their metastases. Also, 9 out of 10 9.2.27-positive patients with clinical stage IV disease presented with progressive disease at the time the blood sample was taken. Three of these patients even had fulminant progression and died within 1–4 weeks. Strikingly, these patients revealed the highest numbers of melanoma cells detected per blood sample. Further studies are needed to assess whether a positive result is a prognostic marker.

The reason for the relatively low percentage of 9.2.27-positive patients in our report even in advanced disease remains speculative. First, the number of circulating cells may be below our detection limit. Second, because the spiking experiments represent an artificial system, the true detection rate and sensitivity of the assay might be lower than expected. Third, intermittent shedding of cells may be the reason for a negative result. Finally, although nearly all human melanoma cell lines examined to date express the 9.2.27 antigen, it might be coated or not present on tumor cells in the bloodstream.

In summary we demonstrate that our immunocytological approach with positive enrichment of tumor cells is a sensitive and user-friendly method to detect melanoma cells in peripheral blood. We could demonstrate that circulating melanoma cells may be detected in a number of patients with advanced disease. The main advantage of our assay, in comparison to PCR techniques, is that not only mRNA sequences but also whole melanoma cells may be identified. Although the method is not suitable for early detection of metastases, it is a valuable tool with which to further investigate phenotypical and biological characteristics of circulating melanoma cells.

ACKNOWLEDGMENT

We are indebted to Prof. Dr. R. A. Reisfeld for kindly providing the mAb 9.2.27 and for helpful discussion of the manuscript.

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