Immunomagnetic Detection of Micrometastatic Cells in Bone Marrow of Colorectal Cancer Patients

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ABSTRACT

Detection of micrometastatic cells in bone marrow (BM) may potentially be of prognostic value in colorectal cancer (CRC). In the present study, we have evaluated our immunomagnetic detection method in model experiments and on BM samples from CRC patients. In repeated experiments, 11 of 12 CRC cell lines consistently bound MOC31 antibody-coated magnetic particles with an average of 98% of the cells being rosetted with the beads. When different numbers of CRC cells (20, 100, 200, and 1000) were admixed to 1 × 10^7 mononuclear cells (MNCs) from BM, a mean of 77% of the cancer cells was recovered. In BM samples obtained from CRC patients at primary surgery, rosetted tumor cells were detected in 46 of 275 samples (17%) upon screening of 2 × 10^7 MNCs/sample. The fractions positive were: 10% (5 of 49) in Dukes' A; 17% (20 of 115) in Dukes' B; 23% (18 of 78) in Dukes' C; and 9% (3 of 33) in Dukes' D. Of 206 control samples, three (1.5%) contained cells in BM that formed rosettes with the MOC31 beads. In positive samples, a median of eight tumor cells (range, 2–120) were identified per 20-μl examined fraction, representing about one-tenth of the total sample. The results demonstrate the feasibility of using the immunomagnetic method for detection of micrometastatic CRC cells. Furthermore, that screening of 2 × 10^7 MNCs in a BM sample can be completed in <3 h makes the method an attractive alternative to other techniques.

INTRODUCTION

Thirty to 60% of patients with CRC undergoing primary surgery with curative intention (R0 procedure) still die from metastatic disease. Consequently, it is assumed that tumor cells, not detected preoperatively, still remain in these patients. Over the last two decades, substantial research efforts have been invested in detecting occult (micrometastatic) tumor cells in different tissue compartments, such as blood, BM, and lymph nodes. Data from such studies could potentially give more accurate prognostic predictions. Sensitive methods for detecting micrometastases have been developed, and for several tumor types, the presence of micrometastatic cells in BM has been shown to correlate with a poor clinical outcome (1). Whether this also applies to CRC is not established, and more data from large and unselected patient populations are needed before examination of circulating CRC cells can be recommended as part of the clinical routine preoperative work-up (2).

Immunocytochemistry and RT-PCR are the methods most commonly used for micrometastasis detection (3), but questions may be raised about the specificity and sensitivity of both techniques. We have developed an immunomagnetic method for the detection and isolation of tumor cells from different tissue compartments (4–6) and adapted it for the detection of CRC cells in BM samples. We used superparamagnetic, 4.5-μm beads (Dynabeads M450; Dynal, Oslo, Norway) coated with the MOC31 monoclonal antibody. This antibody recognizes the Ep-CAM antigen, which is consistently present on the surface of most epithelial cells and highly expressed in CRC. The method allows several logs of enrichment of the cell population of interest, permitting screening of a large number of live cells. This results in highly sensitive detection and facilitates further characterization of the selected micrometastatic cells. In this study, the immunomagnetic method for CRC was validated in model experiments and applied to fresh BM samples obtained from 275 CRC patients undergoing primary surgery.

MATERIALS AND METHODS

Cell Lines. Twelve CRC cell lines were used in binding profile experiments. The cell lines Co205, HCT15, HCT116, Colo320DM, SW480, SW620, CaCo2, HT29, and WiDr were

1 The abbreviations used are: CRC, colorectal cancer; RT-PCR, reverse transcription-PCR; Ep-CAM, epithelial cell adhesion molecule; BM, bone marrow; HSA, human serum albumin; MNC, mononuclear cell.

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all purchased from American Type Culture Collection (Manassas, VA). The cell lines KM20L2 and HCC2998 were kindly provided by Michael R. Boyd (National Cancer Institute, Frederick Cancer Research Facility, Frederick, MD). Co115 cells were obtained from B. Sordat (Epalinges, Lausanne, Switzerland). All cell lines were negative for Mycoplasma infection.

**CRC Patients.** Patients undergoing surgery for assumed or verified CRC were included in the study. The study was approved by the Regional Ethics Committee (Health Region II, Norway), and patient informed consent was obtained. BM aspirates from 316 patients recruited from five hospitals in the Oslo region were analyzed at the time of primary surgery. Forty-one patients were excluded for the following reasons: not invasive cancer (n = 25); insufficient material for analysis (n = 2); previous epithelial cancer (n = 7); histology other than adenocarcinoma (n = 5); or radiation or chemotherapy before surgery (n = 2). The study population thus included 275 patients (128 women, 147 men; median age, 72 years (range, 21–98 years]). Eighty-seven (32%) of the tumors were localized in the rectum and 188 (68%) in the colon. The histological assessment of the resected specimens was performed in four pathology laboratories. To ensure a consistent staging and grading, a reference pathologist reevaluated all reports and primary sections. The stage distribution is summarized in Table 1.

**Malignoma Control Material.** In a separate project, BM aspirates from 176 patients with malignant melanoma with different stages of disease (51 women, 125 men; median age, 57 years (range, 20–92 years)] were examined. Parallel samples of BM were processed using MOC31 as a control antibody.

**Conjugation of Magnetic Beads.** Dynabeads M450 rat antimouse IgG1 or M450 sheep antimouse IgG (Dynal) were suspended in 1 ml of PBS with 1% HSA (Octapharma AG, Ziegelbrücke, Switzerland), and 2 μg of MOC31 antibody (IQ Products, Groningen, the Netherlands) was added per mg beads. The suspension was incubated at 4°C for 30 min, followed by washing in cold PBS with 1% HSA for removal of excess antibody. PBS with 1% HSA was added to make a final concentration of 2 × 10⁸ beads/ml. Uncoated beads for control experiments were prepared similarly without adding the primary antibody.

**Binding Profile Studies with MOC31-coated Beads.** Cell lines were cultivated in RPMI 1640 with 10% FCS under recommended conditions, and the cells were detached with trypsin-EDTA (all products from Life Technologies, Inc., Paisley, United Kingdom). Cell suspensions were prepared with an average of 5 × 10⁶ cells in 1 ml of PBS with 1% HSA. Magnetic immunobeads with or without conjugated antibody were added at a ratio of 20 beads to 1 tumor cell, ensuring an excess of beads. At least three experiments were performed per cell line at different time points. Cell suspensions (1 ml) were incubated at 4°C for 30 min, followed by light microscopic examination of 20-μl fractions of the suspension. The percentage of bead-rosetting cells was determined by counting cells with and without five or more beads attached to each cell.

**Sensitivity Experiments.** Previously stored MNCs isolated from BM samples obtained from healthy donors were thawed using DNase I (Roche Diagnostics, Mannheim, Germany) in the thawing medium to prevent aggregation of cells. Various numbers of tumor cells from the cell line Co205, obtained by consecutively diluting a cell suspension with a known concentration of cells, were added to 1 × 10⁷ MNCs in round-bottomed, 10-ml test tubes. For each experiment, four parallels were processed, each containing 20, 100, 200, and 1000 tumor cells. M450 rat antimouse IgG1 immunobeads coated with MOC31 antibody were added in a ratio of one bead to two MNCs in a total volume of 1 ml. The samples were incubated at continuous rotation at 4°C for 30 min, diluted in cold PBS with 1% HSA, and exposed to a strong magnet for 1–2 min to separate bound “rosetted” and unbound cells. The supernatant was decanted with the tubes still in the magnet holder, and the remaining fraction was resuspended in 200 μl of PBS with 1% HSA. Fractions of 20 μl each were then examined in a light microscope, and bead-rosetting cells were counted in the entire sample.

**Detection of Micrometastatic CRC Cells in Patient Samples.** A total average of 20 ml of BM was drawn from both anterior iliac crests. MNCs were separated by gradient centrifugation using CPT tubes (Becton Dickinson Co, Franklin Lakes, NJ) and washed once in PBS with 1% HSA. Two × 10⁷ MNCs were added to 10-ml, round-bottomed test tubes, and magnetic immunobeads with or without conjugated antibody were added at a ratio of one bead to two MNCs in a total volume of 1 ml. The samples were incubated at continuous rotation at 4°C for 30 min, diluted in cold PBS with 1% HSA, and the rest of the procedure was carried out as described above (Fig. 1). Total sample processing time ranged between 2 and 3 h. A sample was classified as positive if a minimum of two cells rosetted at least five beads with the MOC31 antibody, and no rosettes were observed with the control beads.

**Processing of Control Malignoma Samples.** BM samples were drawn under local anesthesia from the posterior iliac crest of patients with malignant melanoma. In these samples, MNCs were separated by Lymphoprep centrifugation (Nycomed, Oslo, Norway) and washed once in PBS with 1% HSA. Two × 10⁷ MNCs were used for each experiment, but if the procedure yielded a small amount of MNCs, 1 × 10⁷ cells were used. The MNCs were added to 10-ml, round-bottomed test tubes, and M450 sheep antimouse IgG beads coated with MOC31 antibody were added at a ratio of one bead to two MNCs in a total volume of 1 ml. The rest of the procedure was carried out as described above.

**RESULTS**

**MOC31 bead Rosette Formation with CRC Cell Line Cells.** In repeated experiments, 11 of the 12 cell lines consistently bound MOC31 beads, with a mean of ~98% of the cells

<table>
<thead>
<tr>
<th>Dukes’ stage</th>
<th>No. of patients (%)</th>
<th>No. of positive samples (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>49 (18%)</td>
<td>5 (10%)</td>
</tr>
<tr>
<td>B</td>
<td>115 (42%)</td>
<td>20 (17%)</td>
</tr>
<tr>
<td>C</td>
<td>78 (28%)</td>
<td>18 (23%)</td>
</tr>
<tr>
<td>D</td>
<td>33 (12%)</td>
<td>3 (9%)</td>
</tr>
<tr>
<td>All stages</td>
<td>275 (100%)</td>
<td>46 (17%)</td>
</tr>
</tbody>
</table>
being rosetted (Fig. 2). The rosetting pattern was relatively uniform for all these cell lines, with between 5 and 7 beads/cell to complete coverage of the cells with beads. The results for the WiDr and CaCo2 cell lines showed, however, higher inter-experiment variation (SD of 10 and 15, respectively) than for the other cell lines. In Colo320DM, only 32% of the tumor cells were rosetted, indicating a low expression of the target antigen in this cell line.

**Recovery of Co205 Cells Admixed to MNCs.** In six different experiments with tumor cells added to normal MNCs, the mean fraction of Co205 cells recovered was 77% (Fig. 3). The recovery varied somewhat with the number of target cells in the sample. Thus, when 20, 100, 200, or 1000 cells were added, the mean number of recovered cells was 20 (SD 5), 63 (SD 18), 128 (SD 42), and 786 (SD 200), respectively. Importantly, these results demonstrate a high recovery rate with low numbers of target cells present among $1 \times 10^7$ nontarget cells.

**Detection of Rosetted Cells in Clinical Samples.** In the total group of CRC patients, 17% (46 of 275) had rosetted cells in their BM samples, irrespective of disease stage (Table 1). The
frequency was 10% (5 of 49) in Dukes’ A, 17% (20 of 115) in Dukes’ B, and 23% (18 of 78) in Dukes’ C stages, whereas the proportion in the Dukes’ D group was 9% (3 of 33). The overall median number of rosetted cells in the positive samples was 8 (range, 2–120). Of the 46 positive samples, 35 had >5 detected cells, 19 had >10 cells, and 14 had >15 cells (Fig. 4). This represents the number of rosetted cells found in one 20-μl fraction examined per sample, or the average number detected when more than one such 20-μl fraction was examined. These numbers represent about one-tenth of the total number of immunobead-selected cells present in the entire sample. There was no correlation between the number of rosetted cells and stage of disease.

In the control melanoma group, cells rosetted with MOC31 beads were found in only 1 of 176 patient samples. This demonstrates the high specificity of the MOC31-coated beads for cells of epithelial origin.

Thirty patients undergoing surgery for suspected CRC were found to have diverticular disease (n = 7), colorectal adenomas (n = 18), and other histological types of cancer (n = 5). Two of these patients had MOC31 bead-rosetted cells in BM, of which 1 had 50 rosetted cells in one 20-μl fraction; the other had 8 cells. Both of these patients had colorectal adenomas with carcinoma in situ (Tis) at histology. Together, the results show that of a total of 206 non-CRC individuals screened with this method, 3 patients (1.5%) had positive findings in their BM.

**DISCUSSION**

The present study has documented the feasibility of our immunomagnetic assay for detecting micrometastatic cells in BM samples from CRC patients. In model experiments, a mean of 98% of the cells bound at least five beads to their surface in 11 of 12 CRC cell lines. Moreover, a consistently high recovery of target cells was achieved when different numbers of Co205 cells were mixed with 1 × 10^7 MNCs from BM and isolated with immunomagnetic beads, even when very small numbers of tumor cells were added. Furthermore, rosetted tumor cells were detected in 17% of BM samples obtained at primary surgery from 275 patients with CRC with a median of eight cells isolated per positive sample.

The overall frequency (17%) of positive samples from the CRC patients examined in the present study is lower than in many of the published reports using other methods. Thus, studies performed using immunocytochemical techniques report overall frequencies between 27 and 32% positive samples in population sizes of between 88 and 277 patients (7–9). With RT-PCR, <20 clinical studies, using several different markers (CK19, CK20, CEA, GCC, and CD44), have been published on detection of CRC cells in blood and BM, and most of these report data obtained on material from relatively small patient populations, commonly <100. The fraction of positive cases ranges between 0 and 40% for BM samples, and for blood the variation is even more pronounced (10–74%; Refs. 10–15). Because the study designs differ, as do the assays, such inconsistency is not unexpected.

Although the incidence of micrometastatic disease in the present work is lower than that reported in most other studies, the increasing fraction of positive samples with stage in Dukes’ A to C is in agreement with data published previously (9, 13, 16). We found, however, a relatively low frequency of positive samples in the Dukes’ D population. This could be a stochastic effect, attributable to the small sample size in this group. The prognostic relevance of our findings will be evaluated in a follow-up study, expected to be completed in ~3 years.

Our contention is that the true frequency of positive BM samples from CRC patients cannot be accurately derived from results published previously. Although immunocytochemistry has been considered a standard for detecting micrometastases, considerable inter-observer variation as well as variation between staining protocols has been reported (17). The sensitive RT-PCR technique may show false-positive results because of very low levels of contamination, real or illegitimate transcription of the target gene in nontarget cells, or transcription of pseudogenes (18, 19). Moreover, only one study using other
methods has a population size of >200 patients (8). With our method, a high number of MNCs were screened per sample, the MOC31 antibody is known to bind strongly to CRC tumors and cell lines, and the negative control studies showed a very low number of cases with rosetted cells. These factors suggest that the lower percentage of cases with micrometastases found in the present work represent a reliable detection of true positive cells in the sample. The possibility still exists, however, that some CRC cells with low antigen expression might have been missed with our method.

Similar to any other immunological assay, our method’s accuracy depends on the specificity and sensitivity of the applied antibody. The MOC31 antibody recognizes the Ep-CAM antigen, which has been described by several authors under different names (EGP2, 17-1A, GA733, KS1/4, HEA125, and others), often reflecting the antibody used for detection of the antigen. The protein is expressed on the surface of most human epithelia and their malignant counterparts but not on nonepithelia-derived cells or tissues (20–22). The putative function best documented for this protein is that it is a cell adhesion molecule, but its possible role in tumorigenesis and cancer progression has not been established. The antigen is highly and consistently expressed in CRC and in apical cells of normal colorectal mucosa (23), and an Ep-CAM antibody, 17-1A, has been used in immunotherapy for CRC with a reported 30% reduction in overall mortality (24). Thus, the use of Ep-CAM as target for the detection of circulating CRC cells appeared attractive.

The MOC31 antibody (25) was chosen for our work after comparison of several Ep-CAM antibodies and their binding profiles to tumor cells expressing this antigen, using flow cytometric and immunomagnetic bead techniques. Studies of MNCs from BM with immunocytochemical methods with the MOC31 mAb showed almost no cross-reactivity with normal BM cells (26).

The immunobead method has the advantages of simplicity and speed of processing with a complete sample processing time of 2–3 h. The high number of live cells ($\geq 2 \times 10^7$ MNCs) that easily can be screened increases the sensitivity of the method and should facilitate isolation of large enough numbers of target cells to make further characterization feasible.

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5 Unpublished data.


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