Cellular Proliferation and Prevalence of Micrometastatic Cells in the Bone Marrow of Patients with Clinically Localized Prostate Cancer

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ABSTRACT

The presence of prostate cancer cells in the bone marrow (BM) of patients with clinically localized disease is associated with an increased chance of disease recurrence; however, not all patients develop recurrence. We therefore sought to determine the phenotype of individual micrometastatic cells as a potential method to better predict disease outcome. Immunostaining was performed on BM cells from 46 patients whose BM RNA fraction had been identified to contain prostate-specific antigen mRNA. The prevalence of micrometastatic cells among BM mononuclear cells was determined using an anticytokeratin antibody. Mib-1 antibody was used to determine the percentage of micrometastatic cells that were proliferating. Micrometastatic cells were found in 96% of patient samples, with a 30-fold variation in prevalence ranging from 0.1–3.26/10⁵ BM cells. Prior androgen ablation was associated with a reduced prevalence of micrometastatic cells (P = 0.010). In 68% of patients, some micrometastatic cells were judged to be proliferating at proportions ranging from 1 of 11 (9%) to 4 of 4 (100%). Higher Gleason score of the primary tumor was associated with a higher proliferative proportion of micrometastatic cells (P = 0.038). We conclude that, in patients with clinically localized disease, there is wide variability in the prevalence of micrometastatic cells and the proportion which are proliferating. Long-term follow-up will determine whether the development of clinically obvious metastatic disease is related to higher prevalence of micrometastatic cells in the marrow or the proportion that are proliferating.

INTRODUCTION

Disease recurrence after definitive local therapy for clinically localized prostate cancer most often includes a component of distant metastatic disease (1). Currently, staging modalities are not sufficiently sensitive to detect low volumes of metastatic disease at the time of definitive local treatment. In other words, many patients are understaged, and the majority of distant failures likely occur due to undetected metastatic disease present at the time of therapy (2). The BM³ is the most common site of distant metastasis in prostate cancer. Intuitively, it would seem that direct analysis of BM tissue for metastatic cells (in patients without any other evidence of metastatic disease) may predict the future development of clinically obvious metastatic disease. Prostate cancer BM micrometastasis can be defined as low numbers of cancer cells present in the BM of patients with otherwise localized disease. The clinical significance of these cells is an area of active investigation.

Epithelial cells in the BM can be discriminated specifically on the basis of CK expression. Previously, we demonstrated that the majority of cells with CK(+) cytoplasm in the BM of patients with clinically localized prostate cancer harbor mixed chromosomal aneusomies (3). Therefore, these cells must be cancer cells, and they must be derived from the primary tumor. We and others have used RT-PCR methods to detect prostate cancer-specific mRNA transcripts in the BM (4–8). The presence of transcripts such as PSA mRNA implies the presence of viable prostate cancer cells. We have shown that BM micrometastasis, as defined by presence of PSA mRNA transcripts, is associated with an increased risk of disease recurrence after radical prostatectomy (4).

Although the presence of micrometastatic cells in the BM of patients with clinically localized prostate cancer is associated with an increased chance of disease recurrence, not all patients develop recurrence. Our long-term hypothesis is that characterization of micrometastatic cells beyond their mere presence could lead to a more accurate prediction of the future development of clinically apparent metastasis. In this study, we sought to test the feasibility of determining the phenotype of prostate cancer BM micrometastatic cells. We chose to measure the quantitative prevalence of these cells in the BM, as well as the proportion of these cells in active cell cycle.

MATERIALS AND METHODS

Patients, BM, and RT-PCR. Procedures were approved by the Human Investigations Committee of Wayne State University, and written consent was obtained from each patient. BM

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2 To whom requests for reprints should be addressed, at the Departments of Urology and Pathology, Wayne State University School of Medicine, 540 East Canfield, #9105, Detroit, MI 48201. E-mail: mcher@med.wayne.edu.
3 The abbreviations used are: BM, bone marrow; CK, cytokeratin; PSA, prostate-specific antigen; RT-PCR, reverse transcription PCR.
was aspirated from the iliac crest under anesthesia before surgery or radiation. The mononuclear cell fraction was isolated by density gradient separation. Total RNA was extracted from a portion of the cells, reverse-transcribed, and amplified using primers for PSA, as described previously (6). Southern blotting of the PCR products was used to confirm that the amplified band corresponded to mRNA for PSA. Of 204 consecutive patients with clinically localized prostate cancer undergoing BM aspiration, 46 (23%) had PSA mRNA in the mononuclear fraction of their aspirate by the RT-PCR assay. These 46 patients form the basis for this study. None of these patients had evidence of metastatic disease by serum or imaging studies (Table 1). Forty patients underwent radical prostatectomy, three patients underwent brachytherapy with 125I seed implantation, and three patients had external beam radiotherapy. Six (13%) patients were treated with neoadjuvant androgen ablation therapy.

### Table 1. Clinicopathological characteristics of patient population

<table>
<thead>
<tr>
<th>Clinical stage</th>
<th>Number (%)</th>
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<tr>
<td>cT1c</td>
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</tr>
<tr>
<td>cT2</td>
<td>31 (67)</td>
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<tr>
<td>cT3</td>
<td>3 (7)</td>
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<table>
<thead>
<tr>
<th>Biopsy Gleason score</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1 (2)</td>
</tr>
<tr>
<td>6</td>
<td>30 (65)</td>
</tr>
<tr>
<td>7</td>
<td>15 (33)</td>
</tr>
<tr>
<td>OC</td>
<td>25 (63)</td>
</tr>
<tr>
<td>M</td>
<td>7 (18)</td>
</tr>
<tr>
<td>EPE</td>
<td>4 (10)</td>
</tr>
<tr>
<td>SV</td>
<td>4 (10)</td>
</tr>
<tr>
<td>LN</td>
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<table>
<thead>
<tr>
<th>Specimen Gleason score</th>
<th>Number (%)</th>
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<tbody>
<tr>
<td>5</td>
<td>1 (3)</td>
</tr>
<tr>
<td>6</td>
<td>13 (37)</td>
</tr>
<tr>
<td>7</td>
<td>19 (54)</td>
</tr>
<tr>
<td>8</td>
<td>2 (6)</td>
</tr>
</tbody>
</table>

Note: Median serum PSA 6.4 ng/ml. Twenty-two (48%) patients were African-American.

Micrometastatic cells were detected with an alkaline phosphatase-conjugated Fab fragment of anti-CK monoclonal antibody A45-B/B3 (provided in Epitom Kit; Micromet GmbH, Planegg, Germany). Briefly, slides were fixed in formalin and immersed in 9:1 absolute methanol/hydrogen peroxide to quench endogenous peroxidase activity. Slides were then incubated with a 1:100 dilution of the antibody fragment. After washing, slides were treated with levamisole to quench endogenous alkaline phosphatase activity. Bound anti-CK antibody was visualized with 5-bromo-4-chloro-3-indolyl phosphate-nitroblue tetrazolium. Mib-1 staining was performed by following the manufacturer’s instructions in a kit (Histostain DS Doublestaining Kit; Zymed Laboratories, Inc.). Briefly, nonspecific binding was blocked with normal horse serum, and then slides were incubated with a 1:100 dilution of Mib-1 monoclonal antibody (Immunotech, Marseille, France).

**Analysis.** Microscope slides were scanned systematically at ×20 magnification under transmitted light. Phase microscopy (used to visualize unstained BM cells) and higher power examination were used to confirm positive findings. Cells with a blue cytoplasmic stain were considered to be CK(+). Cells with both a blue cytoplasmic stain and a brown nuclear stain were considered to be CK(+) and Mib-1(+). These cells represented prostate cancer micrometastatic cells in active cell cycle. Cells with a brown nuclear stain only were considered to be Mib-1(+) BM cells.

The total number of cells scanned/slide was calculated by multiplying the average number of cells in a ×20 field by the number of ×20 fields. The prevalence of micrometastatic cells was calculated by dividing the total number of CK(+) cells detected in both single- and double-stain experiments by the total number of cells scanned on all microscope slides. The proportion of micrometastatic cells proliferating (double-stain experiments only) was calculated by dividing the number of CK(+)Mib-1(+) cells by the number of CK(+) cells.

### RESULTS

#### Prevalence of Micrometastatic Cells.

Overall, considering both single- and double-stain experiments, an average of 930,000 mononuclear cells was scanned for each patient. Examples of immunocytochemical staining of control and patient BM slides are shown in Fig. 1. CK(+) cells were easily detected because the dark blue cytoplasmic stain was prominent against an unstained background. Among 46 patients with clinically localized disease whose BM RNA fraction contained mRNA for PSA, CK(+) cells could be found in 44 (96%). The total number of CK(+) cells found in these patients ranged from 1–22. There was more than a 30-fold variation in the prevalence of micrometastatic cells ranging from 0.10–3.26 CK(+) cells/10^5 BM cells (Fig. 2).

Among the 44 samples in which CK(+) cells could be found, an average ± SD of 231,000 ± 217,000 BM cells had to be scanned to find the first CK(+) cell. In the two samples in which no CK(+) cells were found, we estimated that we needed to scan 884,000 cells to ensure that no epithelial cells were present; this number is equal to the average plus three SDs of the number of BM cells that we had to scan to find one CK(+) cell in the 44 samples that were positive. In the two patients in whom no CK(+) cells could be found, we scanned 918,000 and 1,715,000 BM cells. Technical factors associated with the BM aspiration or the RT-PCR assay likely explain our inability to find CK(+) cells in these two samples.

#### Proliferative Status of Micrometastatic Cells.

One or more CK(+) cells were found among BM samples from 38 patients in whom double-staining experiments were per-
formed—this is the group of patients for whom we have cellular proliferative data. CK(+) cells were judged to be proliferating if the nucleus was stained reddish-brown (Fig. 1). Considering all 38 patients together, a total of 200 CK(+) cells was evaluated, of which 71 (36%) were judged to be proliferating [Mib-1(+)]. All CK(+) cells were judged to be Mib-1(-) in 12 of 38 (32%) patients. In this group, an average of 5.2 CK(+) cells/patient was evaluated. One or more CK(+) cells were judged to be proliferating in 26 of 38 (68%) patients at percentages ranging from 1 of 11 (9%) to 4 of 4 (100%). In this group, an average of 6.2 CK(+) cells/patient was evaluated (Fig. 3).

Clinical Correlation. We found that the prevalence of CK(+) cells was significantly lower cells in patients who had undergone androgen ablation therapy before BM sampling (0.4 versus 0.9/10^5 BM cells, P = 0.01). On the other hand, we found no correlation between prevalence of CK(+) cells and serum PSA, biopsy Gleason score, clinical stage, or race. Among 38 patients in whom double-stain data were obtained, we found that the percentage of micrometastatic cells [CK (+)] that were proliferating [Mib-1(+)] was increased in patients whose tumors had Gleason scores ≥7 versus those whose tumors had Gleason scores ≤6 (53% versus 29%, P = 0.038). However, we found no correlation between proliferative status of micrometastatic cells and serum PSA, clinical stage, neoadjuvant androgen ablation, or race. Among the radical prostatectomy patients, there was no correlation between proliferative status and pathological stage.

**Fig. 1** Routine transmitted light and phase contrast images of slides stained for both CK and Mib-1. A, PC3 cells admixed into peripheral blood mononuclear cells (positive control slide). PC3 cells are demonstrated by dark blue cytoplasmic staining for CK. Some cells staining positive for CK also have large brown nuclei, indicating simultaneous positivity for Mib-1. Surrounding blood cells are more easily seen on the phase contrast image of the same microscopic field. B and C, individual cells judged to be both CK(+) and Mib-1(+) found in the BM of two different patients with clinically localized prostate cancer. D, a cell judged to be CK(+) but Mib-1(-) in another patient with clinically localized prostate cancer.

**Fig. 2** Histogram of the distribution of the number of patients with the increasing prevalence of CK(+) cells/100,000 BM cells.

**Fig. 3** Histogram of the distribution of the number of patients with increasing proportions of CK(+) cells that were also Mib-1(+).
DISCUSSION

Presence of occult metastatic disease in patients with clinically localized prostate cancer remains the primary reason for failure of local therapies, such as radical prostatectomy and radiation. It is likely that dissemination already has occurred by the time the patient undergoes treatment of the primary tumor. The BM is the most common site of clinically obvious distant metastatic disease in patients with prostate cancer. Therefore, we (3, 4, 6, 9) and others (7, 8, 10, 11) have proposed direct analysis of BM tissue in an effort to predict the future development of clinically obvious metastasis. These efforts, if successful, would allow avoidance of local therapy and/or early administration of systemic therapy.

Previously, we demonstrated that the majority of prostate cancer BM micrometastatic cells harbor mixed chromosomal aneomies (3). Therefore, these cells are, indeed, cancer cells and must be derived from the primary tumor. We (4, 5, 6) and others (7, 8) have used molecular testing by RT-PCR analysis to indirectly detect the presence of low numbers of prostate cancer cells in the BM. The presence in the BM of mRNA species expressed only by prostate cancer cells (e.g., PSA) indirectly demonstrates the presence of viable prostate cancer cells. We demonstrated that the presence of prostate cancer cells in the BM of patients with clinically localized disease is predictive of serum PSA-detected disease recurrence in the radical prostatectomy population (4).

Although pretreatment RT-PCR status predicts a higher likelihood of disease recurrence, not all patients whose BM is positive develop disease recurrence. At a mean follow-up of 15.4 months after radical prostatectomy, only 26% of RT-PCR-positive patients demonstrated disease recurrence (4). Although the failure rate will undoubtedly increase with longer follow-up, it is likely that some patients will remain disease-free indefinitely. In reviewing this issue, Slovin and Scher (2) concluded that the rates of detection of prostate cancer BM micrometastatic cells in series of patients with localized disease seem higher than historical cure rates of radical prostatectomy. Thus, the mere presence of micrometastatic cells in the BM before local therapy does not guarantee eventual development of grossly evident metastatic disease. In some patients, prostate cancer cells may be present in the BM only transiently. In others, cells may be present, but quiescent.

In an effort to more accurately predict the eventual development of clinically evident metastatic disease, we sought to identify and characterize prostate cancer BM micrometastatic cells by factors beyond their mere presence or absence. We hypothesized that a higher prevalence among BM cells or a higher rate of cellular proliferation would help predict the eventual development of clinically obvious metastatic disease. We used a simultaneous, dual-color immunocytochemical technique. The first color allowed discrimination of cancer cells from BM cells on the basis of cytoplasmic staining, and the second color allowed determination of which micrometastatic cells were in active cell cycle on the basis of nuclear staining.

The micrometastatic burden is quite variable. We found prevalences of micrometastatic cells ranging from 0.1–3.26 cells/105 BM cells. The prevalence was reduced by androgen deprivation therapy before BM aspiration from 0.9 to 0.4/105 BM cells (P = 0.010). This modulation by hormonal therapy suggests that our assay was, at least, semiquantitative and that the measured prevalence was proportional to the true burden of micrometastatic cells in the BM. These data agree with that of Pantel et al. (11), who found that androgen deprivation therapy reduced or eliminated BM micrometastatic cells as detected by immunocytochemistry. Similarly, we found that androgen deprivation therapy reduces the proportion of patients whose BM is positive by RT-PCR analysis (12). Several clinical trials have demonstrated that neoadjuvant androgen ablation before radical prostatectomy fails to reduce disease recurrence. Thus, androgen ablation likely modulates the number of micrometastatic cells without affecting ultimate outcome. It is clear that androgen deprivation status must be taken into account in determining the prognostic value of measurement of micrometastatic burden. Long-term follow-up of patients unaffected by androgen ablation therapy is necessary to determine the prognostic value of this assay.

Our study demonstrated the technical feasibility of determining the phenotype of individual BM micrometastatic cells. The proportion of proliferating cells, as determined by Mib-1 nuclear immunostaining, varied from 0–100%. Significantly, the cellular proliferative rate was higher among patients with higher Gleason scores of their primary tumor. Specifically, the proportion of proliferating micrometastatic cells was 53% in those whose primary tumor had a Gleason score ≥7 and 29% in those whose primary tumor had Gleason score <7 (P = 0.038).

To our knowledge, this is the first study correlating micrometastatic cell phenotype with clinicopathological parameters in prostate cancer. Again, long-term follow-up, together with multivariable analysis, will determine the role of measurement of proliferative proportion of micrometastatic cells. In a similar study of breast and colon cancer BM micrometastasis, Pantel et al. (13) found a correlation between ErbB2 positivity of BM micrometastatic cells and clinical tumor stage. Taken together, these studies demonstrate the need for continued investigation into the phenotype of individual micrometastatic cells in patients with clinically localized disease.

Several technical aspects of our study deserve mention. The study design included a screening step: we stained BM samples only from patients who had PSA mRNA in their BM by RT-PCR. This prescreening allowed us to focus our attention on patients with demonstrated micrometastatic disease and the issue of characterization of phenotype of micrometastatic cells. We used this screening step because the RT-PCR assay can be performed more rapidly and is less labor-intensive than immunohistochemistry for determining the presence of micrometastatic cells (5). Of 204 patients with clinically localized prostate cancer screened by this assay, 46 (23%) were positive. Previously, 45% of a similar patient population were positive by this assay (4). This decrease in the rate of RT-PCR positivity is expected in that the stage and PSA level of patients undergoing radical prostatectomy have decreased markedly in recent years (14).

The anti-CK antibody we used was chosen because of its high sensitivity and specificity. This antibody stains all epithelial tissues (15). Sensitivity experiments using prostate cancer cells seeded into peripheral blood samples demonstrated that we were able to locate individual micrometastatic cells at preva-
lences as low as 1 cell/10⁵ BM cells (data not shown), a rate similar to that reported in other immunostaining studies. In addition, we were able to locate micrometastatic cells in 96% of the BM samples taken from patients with a positive PSA RT-PCR assay. The antibody has also been engineered to provide high specificity. Use of the Fab fragment prevents nonspecific binding to Fc receptors present on some hematopoietic cells. Direct conjugation to alkaline phosphatase avoids false positive staining caused by secondary antibodies. The specificity of this reagent was studied by Borgen et al. (16). At the dilution recommended by the manufacturer, no false positive staining was seen in BM tissue. When a four times higher concentration was used together with longer incubation times, one false positive cell was detected in each of 2 of 15 patients when 2 × 10⁶ cells were scanned (16).

In conclusion, we were able to detect and characterize prostate cancer BM micrometastatic cells by immunohistochemistry. We found a wide range of prevalence and cellular proliferative proportion of these cells. We found that hormonal therapy reduced the prevalence of micrometastatic cells and that a higher cellular proliferative proportion was associated with a higher Gleason score of the primary tumor. These results suggest that phenotypic characterization of micrometastatic cells is feasible. Long-term follow-up of patients with micrometastatic disease will determine whether disease recurrence is related to prevalence in the BM or proliferative capacity of micrometastatic cells.

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