Early Tumor Cell Dissemination in Patients with Clinically Localized Carcinoma of the Prostate

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ABSTRACT

Because a significant number of patients with pathologically organ-confined carcinoma of the prostate subsequently develop recurrent disease, metastasis may occur much earlier than previously believed. We have used a reverse transcription-PCR assay for prostate-specific antigen mRNA and an immunocytochemical staining method for cytokeratins to test this hypothesis in paired peripheral blood (PB) and bone marrow (BM) specimens from 71 patients with clinically localized disease before radical prostatectomy. 14 patients with advanced-stage carcinoma of the prostate, and 30 controls (young healthy volunteers, patients without prostate disease, and patients with benign prostatic hyperplasia). Controls were negative in BM and PB. Fifty-six % of patients with organ-confined tumors (pT2) and 73% of those with extracapsular extension (pT3) were positive in the BM versus 16% of those with pT2 tumors and 27% of those with pT3 tumors in the PB. Patients with advanced-stage disease were positive in 86% of BM versus 71% of PB. The sensitivity of the immunocytochemistry assay to detect tumor cells was lower as compared with the reverse transcription-PCR assay. The results suggest that tumor cell dissemination occurs early during disease progression. Prostate cells seem to preferentially concentrate in the BM rather than the PB, which may be due to sequestration there by homing mechanisms. As the rate of detection in the BM exceeds the proportion of patients with subsequently progressing disease, we hypothesize that only a subset of these cells can survive in the BM and evolve to clinically apparent disease.

INTRODUCTION

Prostate cancer is the most common malignancy in men in the United States and has become the second most common cause of cancer deaths (1). RP is an effective treatment modality for CaP, with excellent survival rates if the cancer is localized to the prostate (2). However, approximately 30% of patients with clinically localized disease at the time of surgery will experience tumor recurrence. These patients may have occult micrometastatic dissemination, which is undetectable by imaging modalities such as radionuclide bone scan, computed tomography, or magnetic resonance imaging. The detection of micrometastases by using immunostaining methods for epithelial-specific antigens such as cytokeratins and/or PSA has been achieved in a variety of cancers including CaP (3-6). Moreover, in two of these studies involving either breast cancer or neuroblastoma, the identification of occult micrometastases had prognostic significance (3, 4).

Several groups have used PSA RT-PCR in the PB of CaP patients before RP and have shown data suggesting that the modality is useful for staging and prognosis (7, 8). Using PSA RT-PCR in BM specimens has also been suggested to improve the accuracy of prostate cancer staging and to identify patients at high risk for metastatic disease (9). To date, no PSA RT-PCR studies have been reported comparing simultaneously obtained PB and BM specimens from patients with CaP.

This article reports the results of studies using a highly sensitive RT-PCR assay to detect PSA mRNA in patients with: (a) clinically localized CaP before RP; (b) advanced-stage CaP; and (c) controls without evidence of CaP. Our results show that BM RT-PCR is more often positive than simultaneously obtained PB. Furthermore, the presence of presumed CaP cells in the BM is an early event because these cells can be detected preoperatively even in many patients with pathologically organ-confined disease.

PATIENTS AND METHODS

Prostate Cell Lines. LNCaP and PC-3 prostate cancer cell lines (American Type Culture Collection, Rockville, MD) were used for sensitivity determinations and as positive controls. The cells were grown in RPMI 1640 (BioWhittaker, Inc., Walk-
ersville, MD) supplemented with 10% fetal bovine serum at 37°C.

**Patient Specimens.** The investigations performed in this study were approved by the Internal Review Board of the University of Washington Medical Centers. The study population consisted of: (a) untreated patients with clinically localized CaP who were scheduled for RP; and (b) patients with advanced stage disease, either newly diagnosed or with treatment failure after therapy. The control population consisted of patients with BPH, patients without prostatic disease undergoing surgery, and young healthy volunteers. Paired BM aspirates and PB samples were obtained from all individuals. Ten-ml samples of BM aspirates were obtained from the anterior or posterior iliac crest (uni- or bilaterally) and drawn into 20-ml syringes containing 10 ml of 6% sodium citrate solution. The BM samples were divided into two equal parts for RT-PCR and ICC. Eight ml of PB were drawn by venipuncture into Vacutainer CPT cell preparation tubes (Baxter Scientific, McGaw Park, IL). The prostate specimens removed at surgery were placed in 10% formalin for at least 24 h and submitted entirely for histological examination in standard cassettes. The left side of the prostate specimens was inked in black and the right side was inked in blue. Definitions were used to determine the stage of the primary tumor according to the second revision of the TNM classification, 4th edition (10): organ-confined tumor (pT2), pT2a (<1.5 cm or ≤ half of one lobe), pT2b (>1.5 cm or ≥ half of one lobe), and pT2c (tumor involves both lobes); and local tumor extension beyond the prostate (pT3), pT3a (unilateral capsular tumor extension), pT3b (bilateral capsular tumor extension), and pT3c (tumor involves seminal vesicle(s)). Grading was evaluated using the Gleason grading system (11).

**RT-PCR Assay.** This procedure has been submitted for publication (12). In brief, 5 ml of BM aliquots were underlaid with 15 ml Histopaque-1077 (Sigma, St. Louis, MO) and spun at 500 RCF for 25 min at room temperature. The cell layers at the interface containing the mononuclear cells were collected, and the volume was brought up to 40 ml with PBS. After removing 10-μl aliquots for cell counts, the remaining cells were centrifuged for 15 min (500 RCF at 4°C). In the PB study, the samples were centrifuged for 30 min (1500-1800 RCF at room temperature). The resulting cell layers at the interface were resuspended in plasma, and the isolation was performed as described for the BM specimens. All of the isolated mononuclear cells from the BM and PB specimens were processed for total RNA extraction using 1 ml of the STAT-60 total RNA isolation reagent (Tel-Test "B", Friendswood, TX). The procedure was performed according to the manufacturer’s instructions.

For generation of first-strand cDNA, an aliquot of 5 μg of total RNA was incubated with 1 μl of random hexamers (0.05 μg/μl; Perkin Elmer Corp., Branchburg, NJ) in a volume of 10 μl. After denaturing at 70°C for 5 min, the RNA primer mixture was cooled on ice for 5 min. A 10-μl reaction mixture [4 μl of 5 × first strand buffer, 2 μl of 0.1 M DTT, 1 μl of 10 mM deoxynucleotide triphosphate mix, 1 μl of RNase inhibitor (10 units/μl; Perkin Elmer Corp.), 1 μl of Superscript II reverse transcriptase (Life Technologies, Inc., Grand Island, MD) and 1 μl of H2O] was added, and the sample was incubated for 5 min at 25°C, followed by 1 h at 42°C. The enzyme was heat-inactivated at 99°C for 5 min. For the PSA PCR, 5 μl of the first-strand cDNA mixture (MIC PCR, 1 μl) and 45 μl (49 μl) of the PCR reaction mixture (5 μl of 10 × PCR buffer, 2 μl of 50 mM MgCl2, 1 μl of each primer (5 μM), 4 μl of 2 mM deoxynucleotide triphosphate mixture, and 1 μl of Taq DNA polymerase (Life Technologies, Inc.) and TAQSTART antibody (Clontech Laboratories, Palo Alto, CA) mixture in a 1:1 ratio and 31 μl of H2O (35 μl) were mixed in an ultrathin-walled PCR tube. The reaction was overlaid with mineral oil and placed in a thermocycler (OmniGene thermal cycler; Hybaid, Middlesex, United Kingdom). PCR parameters were as follows: 80°C for 3 min, 1 cycle; 94°C for 5 s and 60°C for 1 min, 40 cycles (25 cycles); and 72°C for 7 min, 1 cycle. The PCR amplification products were analyzed by agarose gel electrophoresis, resulting in a 460-bp product (550 bp). All primer pairs used were designed to bracket cDNA sequences that in genomic DNA cross an intron-exon boundary. To obtain high specificity, PSA primers were designed from regions of the PSA gene expressing the least homology between PSA and human glandular kallikrein. The PSA primer sequences (synthesized by Integrated DNA Technologies, Inc., Coralville, IA) were as follows: 5′ primer, TTG TGG CCT CTC GTG GCA GGG CAGT; and 3′ primer, TGG TCA CCT TCT GAG GGT GAA CTT GC. A PCR of the housekeeping gene MIC was run as an endogenous external control for each sample. The MIC primer sequences (synthesized by IDT) were: 5′ primer, CAC GTC ATC CAG CAG AGA ATG GAA AGTC; and 3′ primer, TGA CCA AGA TGT TGA TGT AGA AGAG. To confirm the identity of the DNA product generated with PSA primers, the PCR-amplified DNA was digested with restriction endonuclease [0.5 μl of ClaI enzyme (Boehringer Mannheim, Indianapolis, IN), 1.5 μl of 10 × ClaI enzyme reaction buffer, and 13 μl of PSA PCR sample incubated at 37°C for 1 h], yielding two 220- and 460-bp products, if the 460-bp product was originated from PSA.

**ICC.** After aspiration into sodium citrate-containing syringes, 5-ml aliquots of BM were transferred into sterile sodium heparin-containing vacuum tubes. Nucleated cell count was determined, and cell viability was obtained with the trypsin blue dye exclusion assay. Only specimens with a cell viability greater than 50% were used for ICC analysis. The mononuclear cells were isolated by Ficoll density gradient (Pharmacia, Uppsala, Sweden). Cells were washed twice in RPMI 1640 supplemented with 10% fetal bovine serum. Mononuclear cells (2.5 × 10⁴) were resuspended and cytocentrifuged onto glass slides (800 rpm for 1 min). Cytospin preparations were fixed in a paraformaldehyde/Histochoice fixative (Amresco, Solon, OH) and stained using the Vector ABC alkaline phosphatase kit (Vector Laboratories, Burlingame, CA). The substrate used was Vector Red (Vector Laboratories), with levamisol included to block endogenous alkaline phosphatase in the specimen. Slides were counterstained with hematoxylin. The primary antibody mixture consisted of the following monoclonal antibodies: anti-human cytokeratin CAM 5.2 (Becton-Dickinson, San Jose, CA); anti-keratin AE1/AE3 (Boehringer Mannheim); and keratin 8 + 18 + 19, clone NCL5D3 (Monosan).

**Experimental Design of RT-PCR.** Integrity of RNA extracted from BM and PB samples and RT performance were...
monitored by MIC PCR. PSA RT-PCR was run to determine the presence of prostate cells in the clinical specimens. PCR with no cDNA (negative control) and PCR with 10 copies of PSA cDNA (positive control) were run simultaneously. We considered specimens to be truly positive if cDNA was amplified with MIC and PSA primers and if the resulting PSA product was cleaved by restriction enzyme, provided that the negative controls were negative. Samples were assessed as negative if no PSA product was detected, but MIC PCR and positive controls were positive. Any detection of PSA mRNA in BM or PB samples was counted as a positive RT-PCR result.

**Experimental Design of ICC.** For an ICC to be valid, the PC-3 positive control must show intense immunostaining with the anti-cytokeratin mixture. The patient specimen must show no staining with the negative control mouse serum, and specimen cellularity and morphology must be intact and interpretable. Positive immunostaining results were designated by intense immunostaining of cell cytoplasm and by a cellular morphology consistent with metastatic carcinoma cells. All slides were reviewed in a blinded fashion by at least two observers, and at least three slides/patient sample were analyzed. The detection of any cytokeratin-positive tumor cell was counted as a positive result.

**Statistics.** Differences in the rates of tumor cell detection in the BM and PB were determined using McNemar’s test of concordance and Fisher’s exact test. Student’s t test was used to evaluate the correlation of a positive RT-PCR test with the PSA serum level, Gleason score, and tumor volume of the primary tumor, respectively. The sensitivity, specificity, and PPV and NPV value of the RT-PCR results in the BM and PB were compared with the final postsurgical pathological results (organ-confined, extracapsular disease).

**RESULT**

For this study, the presence of PSA mRNA in the BM will be referred to as micrometastases, and all individuals without evidence of CaP will be referred to as controls.

**Assay Sensitivity.** Sensitivity is defined as the number of prostate cancer cells detectable/10^6 PBMCs, and hereafter, sensitivity is presented as y:10^x. The sensitivity of the RT-PCR assay was 1:10^6 (Figure 1), compared to 1:5 × 10^7 as achieved by ICC.

**Controls.** The control subjects consisted of 10 young healthy volunteers (8 male and 2 female), 13 surgical patients without evidence of prostatic disease, including 8 with other malignancies (e.g., bladder cancer and sarcoma), and 7 patients undergoing transurethral resection of the prostate for pathologically confirmed BPH. BM and PB samples were negative by RT-PCR in all controls (Table 1). BM samples from 20 of these controls were also evaluated by ICC. No cytokeratin-positive cells were detected in any of the control BM samples (Table 2). PB samples were not evaluated by ICC.

**RT-PCR and ICC in Patients with Clinically Localized CaP before RP.** In 32 of 71 patients, 1 BM sample was evaluated by RT-PCR, and in 39 patients, 2 specimens (obtained from the right and left iliac crest) were analyzed. If one side was positive and the other side was negative, the patients were considered to have positive results. Of the 71 patients undergoing RP and bilateral pelvic lymph node dissection, 43 patients had organ-confined PT2 tumors, and 26 patients had PT3 tumors (Table 2). Two patients with clinically unsuspicious pelvic lymph nodes had nodal metastases upon final pathological analysis. Of the BM samples taken from these 71 patients, 44 (62%) tested positive by RT-PCR compared to 14 positive PB samples (20%). When these results were stratified by stage, 24 of 43 BM samples of patients with PT2 tumors (56%) and 19 of 26 BM samples of patients with PT3 tumors (73%) were positive compared to 7 of 43 PB samples (16%; PT2) and 7 of 26 PB samples (27%; PT3), respectively. One of the patients with pelvic lymph node metastases had a negative BM and PB RT-PCR test; the other one was positive in the BM and negative in the PB. Patients who tested positive in the PB were always positive in the BM, with the exception of one patient who was PB positive and BM negative. The difference in the rates of micrometastasis detection in the BM and PB was statistically significant (P = 0.01, McNemar’s and Fisher’s test). For immunocytochemical studies, bilateral BM samples from 48 patients were evaluated. Cytokeratin-positive cells with tumor cell-consistent morphol-
Table 2  RT-PCR and ICC results in 30 controls and 71 patients before RP stratified by pathological stage

<table>
<thead>
<tr>
<th>Pathological stage</th>
<th>Patients (n)</th>
<th>Positive RT-PCR in BM (%)</th>
<th>Positive RT-PCR in PB (%)</th>
<th>Positive ICC in BM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>0/20 (^a)</td>
</tr>
<tr>
<td>Total</td>
<td>71</td>
<td>44 (62) (^b)</td>
<td>14 (20) (^d)</td>
<td>5/48 (^b) (10)</td>
</tr>
<tr>
<td>pT2N0 (total)</td>
<td>43</td>
<td>24 (56)</td>
<td>7 (16)</td>
<td>3/31 (10) (^e)</td>
</tr>
<tr>
<td>pT2a</td>
<td>11</td>
<td>3 (27)</td>
<td>1 (10)</td>
<td>0</td>
</tr>
<tr>
<td>pT2b</td>
<td>5</td>
<td>2 (40)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pT2c</td>
<td>27</td>
<td>19 (70)</td>
<td>6 (22)</td>
<td>3 (10)</td>
</tr>
<tr>
<td>pT3N0 (total)</td>
<td>26</td>
<td>19 (73)</td>
<td>7 (27)</td>
<td>2/17 (^d) (12)</td>
</tr>
<tr>
<td>pT3a</td>
<td>14</td>
<td>10 (71)</td>
<td>4 (29)</td>
<td>1 (6)</td>
</tr>
<tr>
<td>pT3b</td>
<td>8</td>
<td>6 (75)</td>
<td>2 (25)</td>
<td>1 (6)</td>
</tr>
<tr>
<td>pT3c</td>
<td>4</td>
<td>3 (75)</td>
<td>1 (25)</td>
<td>0</td>
</tr>
<tr>
<td>pT(\infty)+ (total)</td>
<td>2</td>
<td>1 (50)</td>
<td>0</td>
<td>NA (^f)</td>
</tr>
</tbody>
</table>

\(^a\) For ICC, 20 controls and 48 patients with clinically localized CaP were analyzed.
\(^b\) \(P = 0.01\) (McNemar’s and Fisher’s test, respectively).
\(^c\) NA, not applicable.

Fig. 2  Immunocytochemical detection of tumor cells. Cytokeratin immunostaining of tumor cells shows intense immunolabelling reaction, whereas hematopoietic cells show no immunostaining reaction. Original magnification, ×200.

Table 3  Correlation of the serum PSA level, the Gleason score, and the tumor volume of the primary tumor and the presence of micrometastases in BM (range, mean, SD) \(^*\)

<table>
<thead>
<tr>
<th>Serum PSA (ng/ml)</th>
<th>Gleason score</th>
<th>Tumor volume (cc)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM+ ((n = 44))</td>
<td>2.11–22.21</td>
<td>5–9</td>
</tr>
<tr>
<td>BM– ((n = 27))</td>
<td>1.66–15.55</td>
<td>5–8</td>
</tr>
</tbody>
</table>

\(^*\) Differences in mean serum PSA, Gleason score, and tumor volume between patients with positive (BM+) and negative (BM−) RT-PCR tests were statistically not significant \((P > 0.05, \text{Student’s } t\)-test). There was also no correlation between these parameters and the risk of having a positive PB test \((P > 0.05)\).

Table 4  Correlation of RT-PCR with pathologically extracapsular disease (organ-confined, \(n = 43\); nonorgan-confined, \(n = 28\))

<table>
<thead>
<tr>
<th>BM RT-PCR</th>
<th>PB RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>True positives</td>
<td>20</td>
</tr>
<tr>
<td>True negatives</td>
<td>19</td>
</tr>
<tr>
<td>False positives</td>
<td>24</td>
</tr>
<tr>
<td>False negatives</td>
<td>8</td>
</tr>
<tr>
<td>NPV (%)</td>
<td>70.4</td>
</tr>
<tr>
<td>PPV (%)</td>
<td>45.5</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>71.4</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>44.2</td>
</tr>
</tbody>
</table>

We determined the association of micrometastases in the BM with the preoperative serum PSA value, the tumor volume, and the Gleason score of the surgical specimen (Table 3). Mean serum PSA and tumor volume in patients with a positive BM test were slightly higher compared with those of patients without micrometastases; however, the differences in mean serum PSA, mean tumor volume, and mean Gleason score in patients with a positive or negative preoperative BM RT-PCR were not statistically significant \((P > 0.05\) for all combinations, Student’s \(t\)-test). There was also no correlation between these parameters and the risk of having a positive PB test \((P > 0.05)\).

Comparison of Pre- and Postoperative RT-PCR Results. We compared the results of BM RT-PCR before and after RP in 24 patients (Table 5). Postoperative BM and PB samples were obtained between 1.5 and 12 months after surgery (mean, 4.6 months). In seven patients, the RT-PCR results remained negative. Six of seven patients (86%) had organ-confined disease, of whom one had an elevated postoperative serum PSA of 0.9 ng/ml 11 months after surgery, and the other had a pT3a lesion upon pathological evaluation (serum PSA,
To address this question, we compared paired BM and PB samples by BM and PB RT-PCR. Three of three patients were negative by BM RT-PCR before therapy to a positive test after therapy. Only 1 of 21 patients showed a conversion from a BM RT-PCR test before and after surgery had organ-extended tumors; of these, one had an elevated serum PSA (0.21 ng/ml) 10 months after surgery. Thus, 4 of 18 (22%) patients with a negative postoperative BM test had non-organ-confined tumors. Four of six patients (67%) with a positive BM RT-PCR test before and after surgery had organ-extended tumors; of these, two patients had elevated serum PSA levels 2 months after surgery (0.2 and 10.8 ng/ml, respectively). So far, we have not seen a conversion from a BM RT-PCR negative result before therapy to a positive test after therapy. Only 1 of 21 concomitantly drawn PB samples remained positive; this patient also had a positive postoperative BM test. Three other PB samples were technical failures.

**RT-PCR and ICC in Patients with Advanced-Stage Disease.** Fourteen patients with advanced-stage CaP underwent unilateral BM biopsies that were evaluated by RT-PCR. Ten of the 14 patients were also evaluated by ICC. Eleven of these patients had prior treatment and a subsequent tumor recurrence or disease progression as measured by serum PSA or radiological recurrence. In 3 of these 14 patients, CaP was newly diagnosed without having prior treatment, but with clinical evidence of lymph node and/or bone metastases. As shown in Table 6, 9 of 11 patients (82%) with evidence of tumor progression were positive by BM RT-PCR (PB RT-PCR, 7 of 11 (64%); ICC, 2 of 7 (29%). All four patients (100%) with positive bone scans suggestive of bone metastases tested positive by BM and PB RT-PCR. Three of three patients were positive by ICC (100%).

**Role of Cell Number and Biopsy Site.** An issue of interest was whether the higher number of mononuclear cells obtained with a BM biopsy was the primary reason that there were more positive RT-PCR results in the BM than in the PB. To address this question, we compared paired BM and PB specimens with equal numbers of mononuclear cells (4–10 × 10^5 cells) in a subset of six consecutive patients. The cells were processed, and aliquots of total RNA were analyzed by RT-PCR. We found four of six patients with a positive BM RT-PCR, whereas the paired PB samples tested negative. Two of the six patients had negative BM and PB RT-PCR results.

In a subset of 39 consecutive patients, we examined the influence of a unilateral vs. a bilateral BM aspiration biopsy on the frequency of a positive BM RT-PCR test (Table 7). We found different results (one side positive, one side negative) between right- and left-sided biopsies in 12 of 39 patients (31%). If only one side had been biopsied, the frequency of positive results would change from 74% (29 of 39 patients) to 57% (22 of 39), which is still considerably higher than in the PB. There was no correlation between the side distribution of tumor in the prostate and the side distribution of micrometastases found in the iliac crest BM biopsy (data not shown).

**DISCUSSION**

Because prostate cancer metastasizes primarily to bone, there has been great interest in detecting BM micrometastases to improve staging and to understand metastatic mechanisms. In 1949, using routine pathological analysis, Rundles and Jonsson documented micrometastatic disease in BM biopsies of men with early stage prostate cancer who had no radiographic evidence of skeletal disease (13). Subsequently, several groups observed prostate cancer cells by routine pathological examination in BM biopsies from men with apparently localized CaP, and BM biopsies were proposed for use in routine staging until it was realized that the test lacked adequate sensitivity and rarely provided unique clinical information (14, 15). Recently, several investigators using ICC analysis of BM cells found apparent prostate (or at least epithelial) cells in 13–33% of patients without nodal or distant metastases (5, 6, 16, 17). These studies utilized antibodies to either cytokeratins or PSA. No follow-up data are available to determine the prognostic value of this approach.

In 1994, Wood et al. published the first study using the sensitive RT-PCR technique for the detection of PSA mRNA in the BM of CaP patients (n = 55), with a sensitivity of 1:10^6 (9, 18). They observed a positive test in the BM samples of: 20% of patients with pathologically organ-confined disease (pT2), 65% of those with extraprostatic disease, and 83% of the patients with nodal or distant metastases. Subsequently, Wood et al. reported on the follow-up of 79 patients with clinically localized CaP who underwent BM biopsy before RP (19). Whereas 5% of the patients with a negative PCR test had a biochemical recurrence (serum PSA level greater than 0.4 ng/ml), 21% of the patients with a positive preoperative PCR test relapsed, and the disease-free survival in this group was significantly shorter. The authors concluded from these studies that the detection of micrometastases in the BM of CaP patients may improve the accuracy of staging and identify patients at high risk for developing metastatic disease.

Our studies extend these observations by directly comparing paired BM and PB samples from individual patients, both pre- and postoperatively. Using a PSA RT-PCR assay with a sensitivity of 1:10^6, we observed a positive test in the BM samples in 55% of the patients with organ-confined disease, 78% of those with extraprostatic disease, and 86% of patients with nodal and/or bone-metastatic disease. Analysis of the corresponding PB samples showed less frequent positive PCR results (16, 26, and 71%, respectively). The higher positivity rates in the BM of patients with localized tumor compared with Wood’s data may be explained by our 100-fold higher assay sensitivity. The likelihood that the increased positivity is due to

<table>
<thead>
<tr>
<th>Table 5</th>
<th>Serial comparison of BM RT-PCR results in 24 patients before and after RP</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM RT-PCR (pre/post RP)**</td>
<td>No. of patients</td>
</tr>
<tr>
<td>Neg./neg.</td>
<td>7</td>
</tr>
<tr>
<td>Pos./neg.</td>
<td>11</td>
</tr>
<tr>
<td>Pos./pos.</td>
<td>6</td>
</tr>
<tr>
<td>Neg./pos.</td>
<td>0</td>
</tr>
</tbody>
</table>

**Neg., negative; pos., positive.**
254 PSA RT-PCR in BM and PB

The anti-cytokeratin mixture was chosen because metastatic extracapsular disease is caused by a relatively high detection rate of tumor cells in the BM, which is higher than in the PB in patients with breast cancer rather than in PB has been found in other solid tumors (20-24). The increased prevalence of tumor cells in the BM is higher than that in the PB in patients with breast cancer or neuroblastoma, cancers that incidently have a propensity to metastasize to the BM, as does CaP. We speculated that dissemination of tumor cells to the BM in patients with prostate cancer occurs frequently, but that in most cases, especially with curable tumors, the cells do not survive. Serial data are being collected in these and additional patients to determine the prognostic value of postoperative BM (or PB) RT-PCR.

Patient Initial tx Stage BS Serum PSA (ng/ml) Hormonal therapy BM PB ICC BM

R. S. RP pT3bN1M0 Neg. 33.0 Yes Pos. Pos. Neg.
H. C. RP, XRT pT3bN0M0 Neg. 9.73 No Pos. Pos. Pos.
R. O. RP pT3bN0M0 NA 0.24 No Pos. Neg. NA
L. S. RP, XRT pT3bN0M0 Neg. 4.94 No Pos. Pos. Neg.
L. D. RP pT3cN0M0 NA 5.04 Yes Neg. Neg. Neg.
B. R. XRT + orch. T4N0M0 Neg. 39.94 No Pos. Pos. NA
N. W. CPx pT3cN1M0 Neg. 1.23 Yes Pos. Neg. Neg.
L. C. RP pT3bN1M0 NA 0.47 No Neg. Neg. NA
L. S. PLND pT3cN1M0 Neg. 22.9 No Pos. Pos. Neg.
J. S. PLND pT2N2M0 Pos. 185.4 Yes Pos. Pos. Pos.
L. O. XRT T4N0M1 Pos. 4.8 Yes Pos. Pos. NA
D. K. None T4N0M1 Pos. 314.9 No Pos. Pos. Pos.
C. M. None T4N1M0 Neg. 17.7 No Pos. Pos. Neg.
W. S. None T4N1M0 Pos. 209 No Pos. Pos. Pos.

"Initial tx, initial treatment before RT-PCR assay; stage, pathological or clinical staging at the time of diagnosis or treatment; BS, bone scan at the time of the RT-PCR test; serum PSA, PSA at the time of the RT-PCR test; hormonal tx, hormonal treatment at the time of the RT-PCR test; PLND, pelvic lymph node dissection; CPx, cystoprostatectomy; XRT, external beam radiation; Pos., positive; Neg., negative; NA, not applicable; orch., orchietomy.

Table 6 Clinical data of patients with advanced CaP (n = 14)

Table 7 Bilateral versus unilateral PSA RT-PCR in BM (n = 39)

<table>
<thead>
<tr>
<th>Bilaterally positive</th>
<th>17 (44)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilaterally negative</td>
<td>10 (26)</td>
</tr>
<tr>
<td>Right-side positive only</td>
<td>7 (18)</td>
</tr>
<tr>
<td>Left-side positive only</td>
<td>5 (13)</td>
</tr>
</tbody>
</table>

false positive results is lessened by the fact that all 30 controls tested negative.

In addition, 48 samples were available for ICC analysis. The anti-cytokeratin mixture was chosen because metastatic CaP cells often vary in their PSA expression and may not immunostain with anti-PSA antibodies (6, 18). We found that all patients with a positive ICC result had a positive RT-PCR test. The sensitivity of the ICC method to detect micrometastases was lower as compared with the PSA RT-PCR assay.

In our series, the preoperative RT-PCR positivity rate in the BM increased with more adverse pathological stage and tumor burden. However, the differences in mean preoperative serum PSA, tumor volume, and histological grading between patients who were positive and those who were negative were not statistically significant. Sensitivity and specificity of the BM RT-PCR assay to detect extraprostatic disease were quite low (71 and 44%, respectively), with a PPV of 46% and a NPV of 70%. The low specificity of the BM RT-PCR assay to detect extracapsular disease is caused by a relatively high detection rate of micrometastases in patients with organ-confined disease. Although more follow-up data are needed, it does not seem from our series that a positive preoperative RT-PCR test in the BM is a good predictor of pathological stage in patients with clinically localized CaP before RP. Parenthetically, in this series, PB RT-PCR also did not seem to be a very good predictor, either (sensitivity, 26%; specificity, 84%; PPV, 50%; NPV, 65%).

In contrast to the preoperative BM RT-PCR data, the results of postoperative BM biopsies suggested prognostic value. Patients with positive BM RT-PCR tests postoperatively seemed to have more unfavorable tumor stages. 67% had extracapsular disease versus 22% of those with a negative postoperative BM RT-PCR test. We speculated that dissemination of prostate cells to the BM in patients with prostate cancer occurs frequently, but that in most cases, especially with curable tumors, the cells do not survive. Serial data are being collected in these and additional patients to determine the prognostic value of postoperative BM (or PB) RT-PCR.

Our pre- and postoperative BM and PB RT-PCR results suggest that the BM is nearly always positive when the PB is and is often positive when the latter is not, especially in early stage disease. The increased prevalence of tumor cells in the BM rather than in PB has been found in other solid tumors (20-24). Several groups have shown that the detection rate of tumor cells in the BM is higher than that in the PB in patients with breast cancer or neuroblastoma, cancers that incidently have a propensity to metastasize to the BM, as does CaP. We questioned whether the higher number of cells/BM sample compared to that per PB sample was the reason for the relatively higher BM positivity. We analyzed equal numbers of cells in BM and PB samples in six patients, finding four of six patients with a positive BM test and a negative PB test, whereas two of six patients were negative in both the BM and PB. We concluded that cell number alone did not account for the differences between BM and PB RT-PCR results. However, these data suggest that the detection of micrometastases is strongly influenced by the tissue examined and that prostate cells seem to preferentially reside in the BM rather than the PB.

The positivity rate of BM samples (78%) taken preoperatively in patients with extraprostatic disease (excluding positive lymph nodes) mirrors (like Wood's data) the clinical relapse rate of 50-90% in this group (25-27). However, the finding of micrometastases in the BM of about 55% of patients with pathologically organ-confined disease exceeds by far the reported relapse rates of 12-30% in this group (26, 28). Our data suggest that tumor cell dissemination from the primary tumor
occurs very early and often during cancer progression, even in patients with pathologically organ-confined disease. Possibly, only a few of the escaped cells survive in the BM because of factors in the prostate cancer cell and/or BM milieu. Prostate cancer cells that metastasize to the BM may share some of the characteristics of hematopoietic progenitor cells, such as homing receptors or adhesion molecules. This theory gains further support by the findings of Lang et al. (29). They showed that in long-term BM cultures, malignant prostatic epithelia had a significantly greater colony growth than fibroblasts derived from either benign or malignant prostates or skin. The authors concluded that cell-cell interactions between prostatic epithelia and BM are vital for growth induction.

Several caveats should be discussed. One caveat is the identity of the cells within the BM. One can be reasonably certain that the cells are indeed prostatic in origin because of the several assay and patient controls. However, we have not proven that the prostate cells detected by RT-PCR are really cancerous. Possibly, the architecture of a cancerous prostate gland is altered in such a way that even nonmalignant prostate cells may escape from the prostate or that escape is facilitated by prostate needle biopsy. At present, there is no way to definitively answer this question because prostate cells detected by RT-PCR in the BM are difficult to isolate and/or study. It does seem improbable that normal cells would frequently escape through channels in the prostate “opened up” by cancerous processes or by prostate needle biopsy. In our immunocytochemical studies, we detected cells that looked malignant to routine pathological analysis. Furthermore, other RT-PCR studies in the PB suggest that prostate needle biopsy does not result in a high frequency of cell dissemination. In two studies, Moreno et al. (30) found only 8% of the patients positive after prostate needle biopsy who were negative before the procedure and Cama et al. found a conversion from negative to positive PSA RT-PCR in 6% of the cases (31). Parenthetically, it is unlikely that the closeness of the site of the BM biopsy and the prostate cancer plays a role if the BM RT-PCR assay is positive. Clifton (14) and Mansi (32) reported that there was little difference in the incidence of BM carcinoma cells in random biopsies from the sternum compared to the iliac crest. Whether this remains true when using the more sensitive RT-PCR assay needs to be evaluated. Another caveat is the impact of sampling error on the RT-PCR result. The level of sampling error is difficult to determine because it is not possible to test the entire BM compartment. In our series of 39 patients with bilateral BM biopsies, the detection rate of micrometastases would have been 57% if an unilateral BM biopsy had been performed instead of 74% in the case of a bilateral BM biopsy. It seems likely that the detection rate would increase further if additional BM biopsies were performed.

In addition to the qualitative aspects of tumor cell dissemination (i.e., virulence of the escaping cells and/or environmental factors in the BM) there may also be quantitative factors such as the escape of larger numbers of cells in more advanced tumors. We theorize that RP is a curative procedure not because it removes the prostate before the prostate cells disseminate, but because it removes the prostate before the cells that are continually escaping can change in quantity and/or quality such that they have the potential to lodge more permanently into the BM and eventually grow. If additional clinical RT-PCR observations support this theory, there are several implications. If prostate cancer cell escape is ubiquitous before surgery, and the number of escaping cells is correlated with the development of clinically significant metastases, preoperative RT-PCR is unlikely to yield important information because RT-PCR is not a quantitative test as currently performed. Postoperative BM and possibly PB RT-PCR, especially if serially obtained, may provide important unique clinical information. Furthermore, our results highlight issues that need more attention: (a) are the escaping cells truly cancerous; (b) do the cells concentrate in the BM because of homing mechanisms, or are they just passively filtered and trapped; and (c) what are the important features of the escaped cells and/or the BM milieu that enhance prostate cancer cell survival and growth.

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REFERENCES


Early tumor cell dissemination in patients with clinically localized carcinoma of the prostate.
