Prostate-specific Antigen Messenger RNA Is Expressed in Non-Prostate Cells: Implications for Detection of Micrometastases

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ABSTRACT

Prostate specific antigen (PSA) is generally believed to be expressed only by prostate epithelium. If this were true of PSA RNA, then detecting PSA RNA in cells outside of the prostate would indicate metastasis. PCR can detect rare prostate cancer cells. To enhance sensitivity, we developed "nested primer" PCR to detect PSA RNA. With this method, PSA RNA is present in several non-prostate cell lines, including BG-1 (ovarian), SK-MES-1 (lung), and HL-60 (myeloid leukemia), and some normal blood. A low level of PSA RNA detectable by nested primer PCR is present in some cells of non-prostate origin and may interfere with sensitive methods to detect micrometastases. Transcripts of other genes thought to be organ specific may have similar limitations.

INTRODUCTION

The use of PCR (1) in the detection of human malignant cells depends upon a qualitative difference in gene structure (DNA) or expression (RNA) between the malignant cells and the normal background cells in which the malignant cells reside. In malignancies, particularly hematological malignancies, advantage has been taken of unique chromosomal rearrangements, either physiological such as immunoglobulin or T-cell receptor rearrangement, or pathological such as the Philadelphia chromosome or t(14;18) (2). Specific cytogenetic abnormalities are less common in solid tumors, and in these, the application of PCR has relied on the identification of specific mutations in the tumor cells (3), which permit tumor to be differentiated from normal (4), or tissue-specific genes, which permit detection of genes expressed in abnormal sites to be used to indicate tumor spread to that site (5, 6).

Prostate cancer is the most common malignancy in men. Clinically localized disease accounts for an increasing percentage of cases as screening becomes more widely used. Patients with localized disease may be candidates for radical prostatectomy, radiotherapy, or observation. The optimal choice remains controversial and will vary with the patient's clinical characteristics and personal preferences. Distant relapse rates may be as high as 40% after potentially curative local treatment. Thus, undetectable distant disease was presumably present in at least this group of patients. Identifying such patients might spare them the morbidity of local treatment, and these patients might then be a group in which to test early treatment directed against distant disease. Finding early hematogenous spread would also be instructive as to disease biology, perhaps changing the concept of a time-dependent spread of cancer cells via extension through the capsule, spread to regional lymph nodes, and then to distant sites.

PSA is a glycoprotein synthesized in the prostate epithelium secreted as a normal constituent of seminal fluid. Small amounts are released into the blood, and the measurement of serum PSA levels has proved to be a highly useful tumor marker. PSA protein is normally found in serum, however, and mild elevations are not diagnostic of cancer as they can occur with age and prostatic hypertrophy. PSA has been regarded as synthesized only by prostate epithelial cells, whether normal, hypertrophic, or malignant. Thus, PSA expression would appear to be a tissue-specific target, and detection of expression of PSA within cells that are outside of the prostate should indicate metastatic malignant disease. If PSA regulation occurs at the level of RNA expression, then PCR would be a sensitive and specific means to detect PSA mRNA, and thus PSA-producing cells, in lymph nodes, blood, or bone marrow. This rationale has led to several reports of PSA RNA detection by PCR as a sensitive detection method for cells in regional nodes removed at the time of potentially curative resection (7) or in blood (8-11) and bone marrow (12). The clinical utility of these methods await longer follow-up.

As malignant cells become less differentiated, by definition they express fewer tissue-specific genes, and likely fewer copies of those that are still expressed. When sensitivity of PCR is determined, it reflects the total number of RNA molecules, which in turn reflects the product of the number of abnormal cells and the average copy number of the target. We were concerned that in attempting to detect small numbers of metastatic prostate cancer cells in bone marrow, there might be biological false negatives if few cells were present and each contained low copy numbers of PSA RNA. A "nested primer" method was, therefore, developed to enhance the sensitivity of PSA RNA detection and, it was hoped, obviate the need for hybridization procedures. We report here that cell lines of non-prostate origin as well as normal blood and bone marrow may express low levels of PSA RNA detectable by nested primer PCR. Our results indicate the need for caution in interpreting positive PCR results for PSA RNA, and this may be more generally applicable to what are felt to be tissue-specific genes.

MATERIALS AND METHODS

Cell Lines and Blood and Bone Marrow Specimens. LNCAp, DU145 and PC-3 prostate carcinoma, HCT-8 ileocecal adenocarcinoma, MCF-7 breast cancer, A549 and SK-MES-1 lung cancer, and HL-60 myeloid leukemia cell lines were obtained from the American Type Culture Collection, while BG-1 ovarian cancer cells were provided by Dr. D. E. Saunders (Wayne State University). Each line was grown under standard conditions. Blood and bone marrow samples were placed directly in RNA isolation medium and processed immediately or frozen at -70°C until processing.

RNA-PCR. Total RNA was isolated from cell pellets, fresh or frozen bone marrow, or blood by the one-step acid guanidinium phenol method (RNA-STAT60; Teltest, Friendswood, TX). RNA was pretreated with RNase-free DNase (Ambion, Austin, TX) at a concentration of 1 unit/μl at 37°C for 15 min, then incubated at 95°C for 30 min in the presence of 1 μl RNase inhibitor (USB, Cleveland, OH). cDNA was synthesized from 1 μg RNA using mouse mammary leukemia virus reverse transcriptase primed with oligo(dT) in a 20-μl volume containing final concentrations of 50 mM Tris, 75 mM KCl, and 3 mM MgCl₂ at 37°C for 40 min. For primary PCR reactions, 4 μl of the cDNA reaction mixture were used as template with a final concentration of 5 μM primers, 2.5 mM MgCl₂, and 2.5 units Taq polymerase (PE-Cetus, Norwalk, CT) in 25 μl final volume. Reaction parameters were 94°C for 1 min, 60°C for...
1 min, and 72°C for 90 s for 40 cycles, followed by a 10-min extension at 72°C. For the "nested" PCR, 1 μl of the primary PCR reaction was reamplified in 20 μl final volume with 5 mM primers, 2.5 mM MgCl₂ and 2.5 units Taq polymerase at the same PCR cycle parameters. Precautions to avoid contamination were followed (13), and water controls were set up last in any series of reactions to detect carry-over.

**Primer Sequences.** The primer sequences used were: S116, 5'-GCC TCT CGT GGC AGG GCA GT-3'; S174, 5'-CAT CGT CCC ACT GCA TCA TGA-3'; AS478, 5'-CTC TGG TTC AAT GCT GCC CC-3'; SS555, 5'-GGG TGA ACT TGC GCA CAC AC-3'; and S218, 5'-CGG CAC AGC CGT TTT CAT CC-3'. Primers were synthesized by standard phosphoramidate chemistry at the Wayne State University Core Facility, deprotected, lyophilized, and resuspended in water. From published human sequences of PSA (14), HGK (15), and HPK (16), differences at the nucleotide level for the primers are: S116, 9 of 20 from HGK and 13 of 20 from HPK; AS555, 7 of 20 from HGK and 9 of 20 from HPK; S174, 4 of 20 from HGK and 3 of 20 from HPK; AS478, only 1 of 20 from HGK and HPK. The two sense primers are in exon 2, AS478 in exon 3, and AS555 is in exon 4. The predicted outer amplified product is 439 bp whereas the nested product is 304 bp for cDNA, with an additional 1.6-kb intron 2 if DNA is amplified.

**Analysis of PCR Products.** Eight μl of either the primary or the nested PCR reactions were separated by electrophoresis on 1.5% agarose gels in 0.5 mM Tris-acetate-1 mM EDTA (pH 8). Gels were visualized after ethidium bromide staining under UV transillumination and photographed and then transferred in 1.5 M NaCl-0.4 N NaOH to Nytran membranes (Schleicher & Schuell, Keene, NH). The membranes were UV cross-linked, prehybridized for at least 1 h, and hybridized for 3 h in 750 mM NaCl-75 mM NaH₂PO₄ (pH 7) with 5% SDS at 55°C, and washed in 900 mM NaCl-90 mM sodium citrate with 0.5% SDS at 55°C. The oligo probe (100 ng) was end labeled with T4 kinase and [γ-³²P]ATP (DuPont NEN, Wilmington, DE). The washed Nytran filter was exposed to Kodak-XAR film with an intensifying screen at ~70°C overnight. The 304-bp nested PCR product was directly sequenced with Sequenase (USB, Cleveland, OH) using the S174 primer.

**Cell Lysates.** Cells pellets were frozen at ~80°C and thawed at 37°C three times, debris was pelleted at 2500 rpm for 10 min in a clinical centrifuge, and the supernatant was frozen at ~80°C until assayed by the Hybritech assay according to the manufacturer's directions.

**RESULTS**

Whereas our primary RNA-PCR method for PSA could detect small numbers of LNCaP cells, signals for DU-145 and PC-3 were variable. Whereas these cell lines are described as PSA negative, cell lysates yield detectable PSA by the Hybritech assay (data not shown). In an attempt to ensure that poorly differentiated metastatic prostate cancer cells with few copies of PSA RNA could be detected in blood or marrow specimens, we developed a nested primer method in which a small amount of PCR-amplified PSA cDNA is reamplified by a set of primers internal to the original primers. When an attempt was made to determine the sensitivity of the nested PCR method by extracting RNA from HL-60 cells to which serial dilutions of prostate cancer cells had been added, a signal was generated from the HL-60 RNA alone. Controls without added RNA or without reverse transcriptase were negative; therefore, this was not due to contamination.

**Does the Signal Represent PSA RNA?** Because primers were designed in separate exons so that amplification of cDNA and genomic DNA could be differentiated, thus avoiding problems with the interpretation of signals due to contaminating DNA, processed genes could account for these results. Pretreatment of the RNA with DNase did not remove the signal; therefore, it was not due to processed or pseudogenes (data not shown). To demonstrate that the signal was indeed dependent on RNA, RNA from several cell lines was tested by the nested PCR reaction with and without the addition of reverse transcriptase. No signal at 304 bp is seen in the absence of reverse transcriptase, and the water controls are also negative (Fig. 1). The signals in some of the non-reverse transcribed samples correspond to the expected size of the 1.6-kb intron 2 + 304 bp, and this experiment also rules out a signal due to processed genes. In this figure, in addition to the DU-145 prostate line, the lung-derived A549 and SK-MES, the ovarian derived BG-1, and the myeloid leukemia line HL-60 are positive, while the breast-derived MCF-7 is negative. There are additional signals in Fig. 1 reproducibly visible near the predicted size, in particular, the smaller band in HL-60 present with or without reverse transcriptase and the faint band in MCF-7. PCR was repeated on these cDNA samples and analyzed by both ethidium staining (Fig. 2, top) and after Southern analysis using a PSA-specific oligonucleotide probe (Fig. 2, bottom). This confirms the reproducibility of the PCR reaction signals and that the signal does hybridize with a PSA-specific internal oligo. Also, in HL-60, the larger reverse transcriptase-dependent band but not the lower band present without reverse transcriptase hybridizes with the PSA-specific oligo. Finally, the faint band in MCF-7 is different in size and does not hybridize with the PSA oligo; therefore, MCF-7 is negative for PSA mRNA.

Despite primer design to minimize homology with kallikrein, detection of abundant kallikrein at low efficiency was a possible explanation. While the amplified fragments were of the expected sizes and

Fig. 1. PCR product reflects RNA. Total RNA from the indicated cell lines or no RNA control (H₂O) was placed in duplicate tubes and subjected to reverse transcription (+), or reverse transcriptase was omitted (−). Nested PCR was carried out, and the product was analyzed by 1.5% agarose gel electrophoresis and photography of the ethidium-stained gel under UV transillumination. Lane M, kb ladder (BRL, Gaithersburg, MD).
Fig. 2. PCR products are of predicted size and hybridize to PSA oligonucleotide. The product of nested primer PCR of cDNA from the indicated source was analyzed on 1.5% agarose gel and photographed under UV of the ethidium stained gel (top) or transferred to Nytran and hybridized with 32P-end-labeled S218 oligonucleotide (bottom). Lane 1, water; lane 2, LNCaP; Lane 3, LNCaP, no reverse transcriptase; Lane 4, HL-60; Lane 5, MCF-7.

hybridized to an internal PSA oligonucleotide (Fig. 2), this did not absolutely rule out this explanation. Inspection of the sequences for PSA (14) and the kallikreins (15, 16) reveals a Clal restriction endonuclease site in the amplified PSA PCR segment that is not present in the kallikreins. Digestion of the nested PCR product with Clal cuts both the LNCaP as well as the BG-1 amplified product into two smaller fragments as predicted for authentic PSA (Fig. 3). Direct partial DNA sequencing of the BG-1 nested PCR product confirms that this product is from the PSA gene (data not shown).

**Does the PSA RNA Result in Detectable PSA Protein?** Although not a direct concern for developing an assay for micrometastatic prostate cancer, the finding of promiscuous expression of PSA RNA raised the question of whether these cells actually synthesized detectable levels of PSA. Cell lysates were tested by the Hybritech assay. Extract from 10^7 LNCaP cells in 1.0 ml yielded a PSA value of 5.0 ng/ml. Extracts from HL-60 and BG-1 had no detectable PSA under these conditions.

**Is There Low-level PSA RNA in Blood and Bone Marrow?** Use of the nested RNA-PCR reaction for PSA would only be compromised if the background cells in the tissues of interest, i.e., lymph nodes, blood, and marrow, had detectable levels. We screened samples of blood from 7 normal male and 6 normal female donors for PSA RNA and found that all 13 were positive (Fig. 4). Using 25 cycles in each reaction, 10 of the 13 were positive, with or without hybridization to radiolabeled oligonucleotide probe (Fig. 5). None were positive by standard primary PCR reactions, even after hybridization (data not shown). These findings make interpretation of positive results in such assays questionable.

**DISCUSSION**

The factors that influence the ability to detect micrometastases by RNA-PCR for a tissue-specific gene are the relative number of tumor cells in the sample, the sensitivity of the PCR method, and the RNA copy number/tumor cell and, if any, per background cell. Detection of PSA RNA-expressing cells in non-prostate sites would be a potentially important and clinically useful finding if PSA RNA were only expressed in cells of prostatic origin. Prior reports of RNA PCR for PSA-expressing cells have determined sensitivity based on dilutions of LNCaP cells. Even with reported sensitivities of one LNCaP cell/million background cells, <40% of patients with known metastatic disease had PSA RNA positive cells in the blood detected by standard (8, 9) or nested (10, 11) PCR methods. It is known that prostate cancer cell lines other than LNCaP express lower levels of PSA protein, and PC-3 gives a less intense signal with PSA RNA PCR (Ref. 7; data not shown). Because of our concern that PSA RNA levels may be low in some tumors, as they are in DU-145 and PC-3, and thus clinical sensitivity lower than when cell lines such as LNCaP are tested, and because of the data that even in the presence of documented metastatic disease, sensitivity of detection is not optimal (8–11), we, as have others (10, 11), have developed a more sensitive nested PCR method. Our procedure differs from these others in that we use 40 cycles in each round of PCR and undiluted first round product as template in the nested reaction. We unexpectedly found PSA RNA in non-prostate sources. We believe this is not an artifact because: (a) it represents RNA since no signal is observed at the expected size without reverse transcriptase; (b) It is not kallikrein RNA because the primers sufficiently differ from kallikrein sequences, the bands hybridize with an internal PSA oligo, Clal digestion cuts the PCR product (a Clal site is present in the predicted PSA fragment but not in kallikrein), and sequencing of the PCR product of...
PSA RNA IN NON-PROSTATE CELLS

BG-1 RNA using the PSA primers confirms that the sequence is of the PSA gene; (c) omitting template (water control) yields no bands; therefore, cross-contamination is not present. If PSA RNA-PCR is to be clinically useful in detecting microscopic disease, it must be both sensitive and specific. Our data confirm that nested PCR is a highly sensitive method of detection of PSA RNA. Unfortunately, it appears that low levels of PSA RNA are present in a variety of cell lines and normal cells. Immunocytochemical data have shown PSA cross-reacting material in salivary gland tumors as well as adjacent normal salivary gland tissue (17); PSA has been identified in endometrium (18), and PSA RNA expression has been demonstrated in a subset of breast tumor samples as well as in a hormone-dependent fashion in the T47D breast cancer cell line (19). Therefore, the concept of PSA as being truly prostate specific is somewhat misleading. Even if PSA expression were truly tissue or malignancy specific, prior reports with sensitive PCR methods have found promiscuous transcription of other so-called tissue specific genes (20, 21), so perhaps our finding of PSA RNA in non-prostate cells should not be that surprising.

Previous reports of RNA PCR for PSA have examined a variety of patient populations. Moreno et al. (8) demonstrated the feasibility of this method and found positive cells in the blood mononuclear cell fraction in 4 of 12 stage D patients, including 2 of 4 with pelvic node involvement and 2 of 4 with bone involvement. Controls included 9 males with prostatic hypertrophy and 8 females. Deguchi et al. (7) examined poly(A)-selected RNA isolated from resected cancers and lymph nodes. Their negative controls included blood mononuclear cells from females, two non-prostate cell lines, and bladder and renal cancers. PCR detected metastases in some nodes that were negative by histology and immunostaining. Of 21 patients with clinically localized prostate cancer, 5 had detectable disease in nodes by PCR; however, 3 of these had at least one node positive by histology. Wood et al. (12) compared immunohistochemistry and PCR in the detection of prostate cancer cells in bone marrow mononuclear cells. All 5 patients with known bone metastases, 5 of 7 patients with pelvic node disease, and 19 of 44 patients with clinically localized disease had detectable cells in marrow and, while PCR was more sensitive than immunohistochemistry, 16 of 19 patients with localized disease who had cells detectable by PCR also were positive by immunohistochemistry. Katz et al. (9) examined blood mononuclear cell total RNA by PCR with the products analyzed by ethidium staining or enhanced by addition of digoxigenin-labeled nucleotides and antibody detection on blots. Of 18 patients with positive bone scans, 7 were positive by ethidium staining and another 7 by the blotting method. Of 65 patients with clinically localized disease, 11 were positive by ethidium and 14 by the enhanced method. In view of the data on lymph node metastases detectable by PCR (7), one would need to know whether there were detectable cells in regional nodes by PCR before concluding that these patients had hematogenous spread prior to lymph node metastasis. Seiden et al. (10) found PSA RNA by nested PCR in 6 of 100 patients with clinically localized disease and 11 of 35 with known metastases. These authors discuss potential reasons for the relative insensitivity of the method, including few circulating cells, low copy number of PSA RNA molecules/cell, and the possible hormonally induced alterations in expression as has been described also in breast cancer cells (19). Our data point out that further enhancement of sensitivity may compromise specificity. The data of Israeli et al. (11) on PSA similarly confirm the low yield of positive signals with PSA PCR. It appears from their report that prostate-specific membrane antigen, which is expressed even in anaplastic and hormone refractory cells, may be a more sensitive target for detection. Only longer follow-up will permit determination of whether patients with detectable circulating cells by any method may still be curable with local therapeutic methods. PSA RNA PCR may ultimately be clinically useful; however, as with most medical testing, sensitivity and specificity will not be absolute and...
will need to be balanced. This caveat will likely hold true for attempts to define micrometastases using any non-mutant genetic marker.

REFERENCES


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