Application of Immunomagnetic Beads in Combination with RT-PCR for the Detection of Circulating Prostate Cancer Cells

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Recently published protocols using Reverse Transcriptase Polymerase Chain reaction (RT-PCR) for prostate specific antigen (PSA) provide a sensitive means for detecting circulating prostate cancer cells. Attempts to use these assays for staging of prostate cancer have produced conflicting results. As a first step towards rectifying these discrepancies, a modified immunobead-RT-PCR assay capable of detecting as few as 10 prostate cancer cells in 8cc of blood was developed. This 10 fold increase in sensitivity was achieved in part by introducing two target cell enrichment steps. As a model system to assess sensitivity of the modified assay, template RNA was extracted from PSA positive human carcinoma cells suspended in human

blood and isolated with immunomagnetic beads following incubation with an epithelium specific antibody. After 45 cycles of PCR, product from as few as 10 target cells could be readily detected when displayed on a 2% agarose gel stained with SYBR Green fluorescent dye. The identity of amplified DNA fragments was confirmed by Southern blot hybridization.

When applied to blood samples from patients with proven metastatic disease, the immuno-bead RT-PCR assay was successful in detecting circulating PSA positive epithelial cells, suggesting this assay may be useful for assessment of disease progression or recurrence. *J. Clin. Lab. Anal.* 11:346–350, 1997. © 1997 Wiley-Liss, Inc.

Key words: prostate cancer; metastasis; epithelial antigen; immunomagnetic beads; polymerase chain reaction

INTRODUCTION

Metastasis is the major cause of mortality and morbidity in patients with prostate cancer. In order to monitor disease more accurately several molecular techniques have been recently developed. Most of these rely on the use of RT-PCR with primers specific for prostate specific antigen (PSA) for the detection of circulating prostate cancer cells (CPCC), an approach originally conceived by Moreno et al. (1). This assay or variations developed by other laboratories have been used as a sensitive test to identify patients with local disease expansion, minimal residual or recurring disease (2,3). Similar assays based on mammary or liver associated antigens have also been developed for the detection of circulating cells shed by mammary or hepatocellular carcinomas (4).

RT-PCR assays for detection of circulating tumor cells are based on the assumption that cells expressing tissue specific markers such as PSA are not present in normal blood and are not expressed by peripheral blood cells, thus allowing the use of total RNA extracted from the mononuclear cell fraction of the venous blood in the analysis. The level of sensitivity achieved by RT-PCR analysis of RNA extracted from buffy coats was reported to be approximately 1 PSA positive cell/

10⁵–10⁶ cells (2,3). The clinical utility of RT-PCR assays has been brought into question by conflicting reports from several laboratories using different versions of the original assay. Significant in this regard are two recent studies (5,6) reporting the detection of PSA transcripts in RNA harvested from the peripheral blood of patients without clinically detectable prostate cancer.

In the present report, we describe an improved immunobead-RT-PCR assay with increased sensitivity and specificity for blood borne prostate tumor cells detection and its application to blood samples from prostate cancer patients with clinically proven metastatic disease.

MATERIALS AND METHODS

Cell Cultures

Three human prostate cancer cell lines, LNCaP, PC-3, Du-145, were obtained from ATCC. Cell lines were maintained

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in a humidified 5% CO₂ incubator in RPMI 1640 media supplemented with 5% fetal bovine serum, L-glutamine, 100 U/ml of penicillin and 0.1 mg/ml streptomycin.

Epithelial Cell Specific Antibody

Ber-EP4 (Dako, Glostrup, Denmark), a monoclonal antibody (MoAb) specific to an antigen on the surface of human epithelial cells (7) was used for magnetic bead harvesting of circulating tumor cells at a concentration of 0.5–2.0 µg/10⁶ cells. Binding of Ber-EP4 was assessed by flow cytometry on unfixed suspensions of LNCaP, DU-145 and PC-3 prostate carcinoma cells labeled by an indirect immunofluorescence protocol using FITC conjugated goat-anti-mouse IgG (Sigma) as a secondary antibody.

Blood Samples Collection and Separation of Erythrocytes

Venous blood (8 cc) collected in tubes containing 15 mg EDTA as a preservative was processed within 2 hrs of phlebotomy. Blood diluted 1:1 with phosphate-buffered saline (PBS) was layered over a 1.095 g/ml Percoll gradient (Pharmacia) and centrifuged at 1500 × g for 20 min at room temperature to separate erythrocytes from nucleated cells. Alternatively, nucleated cells were collected in a Vacutainer CPT (Becton Dickinson, #362761) containing 0.1M sodium citrate anticoagulant and a blood separation media composed of a thixotropic polyester gel and Ficoll Hypopaque solution. Similar results were obtained with both methods but the latter method was found to be much quicker and more convenient. In both methods, cells retained at the interface were diluted with cold PBS to a final volume of 4 ml and incubated with epithelium specific antibody as described below.

Target Cells Isolation

The nucleated cell fraction harvested from whole blood was incubated with 1 µg of Ber-EP4 MoAb/10⁷ cells at 4°C for 30 min with constant agitation. After 2 washes in PBS to remove unbound antibody, cells were resuspended in 1 ml PBS/0.1% bovine serum albumin (BSA, Sigma #9418) and transferred into 1.8 ml snap cap cylinder tube (USA/Scientific Plastics, #1420-2700) containing Sheep-anti-Mouse IgG coated M-450 Dynabeads (Dyna, #110.01) prewashed in cold PBS/0.1% BSA. Residual antibody coated cells remaining in the original tube were collected in 0.5 ml PBS/BSA and added to the cell/Dynabead mixture. Optimal recovery of Ber-EP4 antigen positive cells was obtained with a bead:cell ratio of 1:5 in a final volume of 1.5 ml.

After incubation for 1 hr at 4°C with end over end agitation (Labquake rotator) beads and rosetted cells were collected for 2 min with a magnetic MPC6 concentrator (Dyna). Unbound cells were discarded and bead-bound cells were washed with PBS/BSA three times.

Recovery Experiments

In a model study, aliquots containing 10 (three samples), 25 and 50 PSA positive LNCaP cells were mixed with 8 ml of whole blood obtained from a healthy young male volunteer. Prior to gradient separation, tubes were rotated end over end for 20 min at ambient temperature to allow even distribution of the target cells throughout the blood sample. Coulter counter analysis showed that the blood cell mixture contained a total of 8.2×10⁷ white blood cells (WBC) prior to gradient separation and 6.7–6.8×10⁷ WBC after centrifugation. Using immunobeads, target cells coated with Ber-EP4 antibody were isolated and placed into culture. Cells with bound immunobeads and an epithelial cell morphology were visualized by light microscopy and counted after 24 hrs in culture.

RNA Preparation

Total RNA was prepared from cultured LNCaP cells and bead positive or negative cells from whole blood using a “Micro-Scale Total RNA Separator kit” developed by Clontech Labs, Inc (#K1044). Briefly, after the last wash in PBS/BSA, bead bound cells were denatured in a solution containing 4.0 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sodium N-laurylsarcosine and 0.1 M 2-mercaptoethanol. The lysate was vortexed for 30 sec. and spun down for 15 sec. at 13Kxg to pellet the Dynabeads. The supernatant was transferred to a fresh RNase free Eppendorf tube and processed according to the manufacturer’s recommendations. The resulting RNA pellet was resuspended in 30 µL DEPC treated water and stored at –70°C.

RT-PCR

Each RNA sample was analyzed twice by RT-PCR to confirm results. First strand cDNA synthesis: A mixture of 5mM Oligo (dT)₁₂₋₁₈, 50mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂, 20 mM DTT, 125 mM each of four dNTPs and 3 µL of total RNA from bead bound cells was heated at 70°C for 10 min and quickly chilled on ice for 5 min. Droplets of the reaction mixture were consolidated by a brief centrifugation at 12Kxg and 1 µL (200U) of Superscript II Reverse Transcriptase (Gibco BRL) was added. After a 1 hr. incubation at 42°C, cDNA synthesis was terminated by heating at 70°C for 10 min. RNA templates complementary to the cDNA were subsequently removed with 0.5 µL (1 unit) of *E. coli* RNase H (Gibco BRL) digestion for 20 min. at 37°C. cDNAs serving as positive and negative controls for PCR were prepared by the same protocol with 0.25 µg total RNA from the PSA positive cell line LNCaP or with dH₂O.

PCR was performed with oligonucleotide primers based on the PSA cDNA sequence obtained from the GenBank of National Center for Biotechnology Information. Primers were designed using Amplify analysis software (Amplify 1992, Univ. of Wisconsin, Genetics, Madison, WI). Primers specific for a 611 bp segment of PSA mRNA were as follows:

PSA-A (nucleotides 714–733) 5′-TGTACACCAAGGTG-GTGCAT-3′ and PSA-B (nucleotides 1306–1325) 5′-CCATA-GTGAATCAGCTTCCA-3′.

Primers described by Okazaki (8) for a 372 bp fragment of β -actin were: 5′-ACAATGAGCTGCGTGTGGCT-3′ and 5′-TCTCCTTAATGTCACGCACGA-3′.

Oligonucleotides were synthesized in the Department of Pathology, Rhode Island Hospital using an Applied Biosystems Model 392 DNA/RNA synthesizer (Foster City, CA). Following deprotection at 55°C overnight, oligonucleotides were extracted with 1-butanol and resulting pellets resuspended in dH₂O at 10 μ M/ μ L.

Half of the each RT reaction (10 μ L) was used for amplification of cDNAs derived from PSA transcripts. The other half was used to confirm the integrity of the original RNA sample by amplification of β -actin cDNA. The RT reaction was combined with 40 μ L of a PCR master mix to reach the final concentration: 0.2 mM of each dNTPs, 2.0 mM MgCl₂, 50 mM Tris-HCl (pH 8.4), 20 mM (NH₄)₂SO₄, 0.2 μ M of the upstream and downstream primers and 1 U/50 μ L Tfl polymerase (Epicentre Technologies, WI). Amplification was performed in Perkin Elmer 2400 thermocycler using the following schedule: 94°C for 5 min; 10 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 45 sec; and 35 cycles of 94°C for 30 sec, 58°C for 45 sec and 72°C for 1 min; 72°C for 10 min ending at 4°C.

Fifteen microliters of PCR product from the bead bound cells and the negative control and 2 μ L from the positive control were electrophoresed on a 0.5 cm thick 2% NuSieve 3–1 (FMC) agarose gel in 0.045 M Tris-borate, pH 8.3, containing 0.001 M EDTA (TBE). Gels were stained by soaking with gentle agitation for 2 hrs in SYBR Green fluorescent dye (FMC) diluted 1:7500 with TBE. Gels were photographed on Polaroid 667 film over 300 nm U.V. light.

Southern Blot Hybridization

Southern blot analysis was performed to confirm the specificity of PCR products and enhance the sensitivity of detection. RT-PCR with nested primers was used to generate a 485 bp product corresponding to an internal portion (nucleotides 809–1294) of the 611 bp of RT-PCR product amplified from LNCaP RNA templates.

PSA-A′: (nucleotides 809–828) 5′-GGAACCTTGGAATGACCAG-3′

PSA-B′: (nucleotides 1276–1294) 5′-CCCTCTCCTTACTTCATCC-3′

Restriction digestion with AccI endonuclease (New England Biolabs) produced the 111 and 374 bp fragments expected from the authentic PSA-PCR product. Hybridization probe was generated from the 485 bp PCR product by random primer labeling with [α -³²P]dCTP (DuPont, NEN Research Products, Boston, MA, sp. act.=3000 Ci/mmol).

PCR products were electrophoresed on agar gels as de-

scribed above and transferred onto Hybond-N (Amersham, UK) nylon membrane using standard conditions defined by Sambrook and Maniatis. Blots were prehybridized in a Hybridization incubator (Robbins Scientific, Model 1000) for 2 hrs at 65°C in 5 ml of solution containing 6×SSC (900 mM NaCl and 90 mM Sodium citrate), 5× Denhardt's reagent, 0.5% SDS (Sodium dodecyl sulfate) and 100 μ g/ml denatured, fragmented salmon sperm DNA. Hybridization was carried out for 16 hrs at 65°C using 5 ml of a freshly prepared buffer of the same composition containing 1 ng/ml of the radiolabeled 485 kb PSA PCR product (7.5×10⁶ cpm). Blots were washed under high stringency conditions and exposed to X-ray film.

RESULTS

Determination of the Assay Sensitivity

1. Immunobead enrichment recovery experiments

FACS analysis demonstrated that more than 95% of the cells in unfixed suspensions of LNCaP, DU-145 and PC-3 cells were labeled following incubation with Ber-EP4 at a concentration of 1 μ g/10⁶ cells.

Counts after 24 hrs in culture of cells isolated from blood samples seeded with as few as 10 LNCaP cells indicated that greater than 90% of the target cells have been recovered by this procedure. Confirmation that the bead bound cells were LNCaP was made after two weeks in culture by RT-PCR for PSA transcripts using the PSA-A and PSA-B primer set. To determine if the percent recovery had been overestimated because of cell division, counts were made of recovered LNCaP cells plated at a low density after incubation with Ber-EP4 antibody and Dynabeads as described in Methods. The average results obtained from three different experiments showed only a 0.5% increase in cell number during the first 24 hrs in culture.

2. RT-PCR

RT-PCR performed using total RNA from PSA positive LNCaP cells produced the expected 611 bp and 372 bp products for PSA and β -actin, respectively. Identical products were also obtained using total RNA from bead bound cells and isolated from whole blood seeded with as few as 10 LNCaP cells but not from the Ber-EP4 negative fraction (Figs. 1, 2). Southern blot analysis confirmed that the PCR products were derived from PSA transcripts (Fig. 3).

RT-PCR analysis was also performed on bead bound cells isolated from blood samples obtained from 13 patients with proven metastatic, hormone refractory disease. PSA amplicons were detected in 5/6 blood samples collected from patients before initiation of chemotherapy (Figs. 4, 5). In contrast, no PSA amplicons were found in the blood of 5/7 patients who were undergoing hormonal and chemotherapeutic treatment and had responded with a significant decline in serum PSA.

Fig. 1. SYBR Green-1 stained 2% agarose gel showing PSA RT-PCR products generated from transcripts present in whole blood seeded with LNCaP cells. Lane 1: DNA size standards (fX174 RF DNA/Hae III Fragments). Lane 2: Total RNA from cultured LNCaP cells. Lane 3: dH₂O control. Lanes 5-9: Total RNA isolated from LNCaP cells with Ber-EP4 coated magnetic beads: 10, 10, 10, 25, 50 LNCaP cells, respectively. Lanes 10-14: Total RNA isolated from Ber-EP4 negative cells (beads unbound fraction) correspondent to samples 5-9.

However, two patients in this group who failed treatment and did not demonstrate a decline in serum PSA tested positive for PSA transcripts. In addition RT-PCR for PSA was positive in one blood sample from a patient diagnosed 12 years ago with locally disseminated and slow progressing prostate cancer (Figs. 4, 5). Five blood samples obtained from healthy young male donors tested negative in the RT-PCR assay. One sample that failed to yield β -actin PCR product has been excluded from the analysis.

DISCUSSION

In the present study, we have described an immunobead-RT-PCR assay for the detection of circulating prostate cancer cells. This assay is similar in concept to the immunobead-PCR assay described by Hardingham et al. (9), but incorporates an enrichment step for nucleated cells prior to the isolation of circulating epithelial cells with Ber-EP4 MAb and magnetic beads. Using whole blood seeded with PSA positive LNCaP cells as a model system, our assay was capable of detecting as few as 10 tumor cells/8 ml of whole blood, a dilution equivalent to approximately 1 tumor cell/ 7×10^6 nucleated blood cells. When applied to blood samples from

Fig. 2. Parallel amplification of 372 bp b-actin DNA fragment. Samples 1-14 correspond to those in Fig. 1.

Fig. 3. Southern analysis of the PSA PCR amplicons displayed in Fig. 1 with a probe generated by random prime labeling of the 485 bp nested PSA PCR product.

patients with confirmed progressing metastatic disease, PSA positive cells were detected in 5/6 patients.

A number of strategies have been used successfully for the detection of circulating tumor cells. These include assays based on immunomagnetic beads, FACScan analysis, RT-PCR or a combination of these techniques. In the immunobead-PCR assay of Hardingham et al., tumor cells were harvested directly from whole blood instead of the nucleated cell fraction. High cell to bead ratios (20:1) were also used, a requirement that could result from differences between Ber-EP4 levels on colon and prostate tumor cells or from the loss/dilution of beads by the high concentration of red blood cells. When tested on the mixtures of whole blood and colon carcinoma cells, the sensitivity was approximately 70 fold lower than the present assay, but no data was provided indicating whether similar sensitivities were obtained with patient blood samples.

The efficacy of the immunobead enrichment step depends on the uniform expression of the Ber-EP4 epitope by circulating tumor cells and the absence of this epitope on cells of non-epithelial origin. Previous investigations have shown that this antibody is directed against two glycopeptides with mo-

Fig. 4. SYBR Green 1 stained 2% agarose gel: PSA RT-PCR products amplified from male blood donors. RNA templates were extracted from the Ber-EP4 positive cells as described. Mw - DNA size standards (fX174 RF DNA/Hae III Fragments); P - Total RNA from cultured LNCaP cells; N = dH₂O control; M - PSA RT-PCR products from patients with metastatic disease; L - Blood sample from a patient with local tumor dissemination; C - Control samples from men without prostate cancer.

Mw P N - M M M M M M M L C C

Fig. 5. Southern analysis of the samples displayed in Fig. 4 with 485 bp PSA specific probe.

lecular masses of 34 and 39 kDa present on the surface and cytoplasm of all epithelial cells except apical cells in squamous epithelium, hepatocytes and parietal cells (7). FACScan analysis of three different prostate carcinoma cell lines showed that more than 95% of the cells expressed the MAb defined epitope. It remains to be determined whether circulating tumor cells in patient blood samples display the same degree of uniformity, a key factor that could greatly influence the sensitivity of detection.

Sensitivities at frequencies between 10^{-6} – 10^{-7} , a level similar to the immunobead-RT-PCR assay described in this report have been obtained in two recent studies using flow cytometry to detect pre B-cell tumor cells and breast cancer cells, respectively in mixing experiments with nucleated peripheral blood cells. Whether similar sensitivity can be achieved during the analysis of patient samples remains to be determined. An important factor in the detection of rare cells by flow cytometry was the use of both positive and negative markers to eliminate false positive signals resulting from background or nonspecific staining. When compared to multiparameter FACScan analysis, the magnetic bead approach has the advantages of simplicity and low cost. Although it is not technically feasible to sort bead bound cells for expression of a second positive marker, the results obtained with flow cytometry suggest that a negative selection with a marker that is lost with high frequency on prostate cells such as BGPI could provide a further increase in specificity (10). Alternatively, RT-PCR analysis of bead bound cells using primers specific for a second tumor or prostate associated marker such as prostatic alkaline phosphatase or prostate specific membrane antigen could reduce the level of false positives and provide a means for detecting PSA negative tumor cells.

Although the small number of patient samples analyzed in the study did not allow us to apply statistical methods to evaluate the predictive value of the RT-PCR assay, the ability to detect circulating cells in 5/6 blood samples taken prior to therapy from patients with advanced malignancy is encouraging and warrants further investigation. Negative results on patients that responded to therapy with a significant drop in serum PSA levels are consistent with the expected reduction in circulating cells, a conclusion supported by the positive RT-PCR results obtained

from patients that failed to respond to therapy. However, it is also possible that the negative results obtained with some of these blood samples may be unrelated to therapy and instead may reflect an absence of circulating cells or the presence of poorly differentiated, PSA negative cells.

The significance of circulating tumor cells is yet to be determined and a long term follow-up is necessary to address this question. The assay described in this report is technically simple and capable of detecting circulating prostate cancer cells at frequencies close to 10^{-7} . Introduction of an enrichment step for nucleated cells followed by immunobead selection and RT-PCR not only increases the level of sensitivity, but also ensures high specificity of the method, thus reducing the frequency of false results.

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