SHORT TECHNICAL REPORTS

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Immunobead RT-PCR: A Sensitive Method for Detection of Circulating Tumor Cells

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ABSTRACT

Detection of circulating tumor cells and micrometastases in patients with cancer should prove useful in determining prognosis and in planning and monitoring systemic therapies. We have developed immunomagnetic isolation of carcinoma cells followed by reverse transcription polymerase chain reaction (immunobead RT-PCR) as a method for identifying very small numbers of breast cancer cells in blood. The expression of cytokeratin 19 (K19) was used as the marker by which the isolated tumor cells were identified. The immunobead RT-PCR technique allowed detection of one tumor cell per 10^6 leukocytes in whole blood. Immunobead RT-PCR is a highly sensitive method of detecting cancer cells in a hematopoietic environment.

INTRODUCTION

Most common cancers, such as those of the breast and colon, arise from epithelial cells. Mortality in these cancers is not due to the primary cancer but as a consequence of the metastasis of the cancer cells to secondary sites, where they disrupt normal function. Metastasis necessarily involves the entry of cells from the primary tumor into the circulation. We have developed a technique (immunobead reverse transcription polymerase chain reaction [RT-PCR]) that isolates circulating epithelial cells using an epithelial-specific monoclonal antibody bound to immunomagnetic beads and then analyzes the isolated cells using a sensitive RT-PCR assay.

The combination of immunobead isolation of carcinoma cells with RT-PCR of an epithelial mRNA as the tumor cell marker allows this technique to be applied to the majority of carcinomas. This paper describes the development of the immunobead RT-PCR technique for the detection of breast cancer cells in a hematopoietic environment, using expression of cytokeratin 19 (K19) as the marker for the tumor cells. K19 forms part of the cytoskeleton of epithelial cells and is not normally expressed in other cell types including haematopoietic cells (3,15).

MATERIALS AND METHODS

Breast Cancer Cell Lines

The human breast cancer cell lines T-47D, MCF7 and MDA-MB453 (obtained from the ATCC, Rockville, MD, USA) were used in assessing reactivity of the monoclonal antibodies in the optimization of the RT-PCR and in sensitivity experiments. Cell lines were maintained in RPMI 1640 medium (Life Technologies, Gaithersburg, MD, USA) at 37°C in 5% CO_2.

Assessment of Monoclonal Antibodies

Flow cytometry (Epics® Profile II Flow Cytometer; Coulter, Hialeah, FL, USA) was used to assess the efficacy of labeling of the breast cancer cell lines with Ber-EP4 (9), Dako-EMA (7) (both from Dakopatts, Gestrop, Denmark) and the anti-mucin 1 antibody BC-2 (Reference 4; provided by Professor I.F.C. McKenzie, Austin Hospital, Heidelberg, Victoria, Australia). Antibodies were incubated with cell lines at concentrations of 0.5 to 1.25 μg per 10^6 cells. Nine frozen primary tumor samples were assessed for Ber-EP4 staining by immunohistochemistry.

Manipulations of T-47D Cells

Cell monolayers from T-47D were trypsinized and placed in dilute suspension in calcium- and magnesium-free Dulbecco's phosphate-buffered saline (PBS). Under inverse-phase light microscopy, single cells were taken by a micropipet and transferred to the RT-PCR lysis mixture (see RT-PCR section). For experiments to test the sensitivity of the immunobead RT-PCR technique, cells were serially diluted into PBS, and aliquots were transferred to 10 mL of whole blood to give ratios of tumor cells to white blood cells of $1:10^3$ to $1:10^7$.

Table 1. Comparison of Cell Lysis Methods

Nonidet® P-40 (NP40) detergent lysis

Cells in a residual volume of PBS (approximately 2 μL) were suspended in a 9.5-μL volume lysis mixture (see RT-PCR section).

Freeze thawing

Cells in 2 μL suspension were added to a 9.5-μL volume lysis mixture with or without NP40 (Sigma Chemical, St. Louis, MO, USA) and frozen at -80°C. The 9-μL reaction mixture (see RT-PCR section) was added, and the lysate was thawed at room temperature before the initiation of reverse transcription at 37°C.

Heat lysis

Cells in 2 μL suspension were added to an 8-μL volume mixture of 500 ng random hexamers in diethyl pyrocarbonate (DEPC) water and heated at 94°C for 10 min. Twenty units of RNasin® (Promega, Madison, WI, USA) and 10 mM dithiothreitol (DTT; Sigma Chemical) were then added.

Immunobead Isolation of Carcinoma Cells

Immunobeads (2×10^6; Dynabeads® M-450; Dynal, Oslo, Norway), labeled with Ber-EP4 according to the manufacturer's instructions, were added to 10 mL of blood. After incubation at room temperature for 2 h, the tubes were taped to an array of cobalt samarium disc magnets. The beads were washed three times in PBS, during which the unbound cells were washed away. The bead/cell aggregates were then transferred to a microcentrifuge tube for RT-PCR.

Comparison of Cell Lysis Methods

Several cell lysis methods (Table 1) were compared to ascertain which among them most efficiently released mRNA from limiting numbers of T-47D cells for reverse transcription. Reverse transcription was initiated immediately following lysis.

RT-PCR

Cells or immunobead-cell isolates were suspended in a 9.5-μL volume lysis mixture containing 0.3% vol/vol NP40, 500 ng random hexamers (Pharmacia Biotech AB, Uppsala, Sweden), 20 U of RNasin, 10 mM DTT and DEPC-treated water. The RT was initiated by adding 9 μL of reaction mixture to give a final concentration of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, (equivalent to one times first-strand buffer) and 0.5 mM each of dATP, dGTP, dCTP and TTP, 10 mM

DTT and 200 U of SuperScript™ II (Life Technologies). Assuming a residual volume of 1.5 μL associated with the immunobead-cell isolates, the final volume is 20 μL. The entire mixture was incubated at 37°C for 60 min, then the reverse transcriptase was inactivated at 70°C for 10 min.

Seven microliters of the RT product were taken as substrate for PCR in a final volume of 50 μL. The other PCR components were added as a master mix: 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM each of dATP, dGTP, dCTP and TTP, 100 ng of each primer, sterile distilled water and 0.5 U of *Taq* DNA Polymerase (Boehringer Mannheim, Mannheim, Germany). The primers for K19, which span several introns and do not amplify the K19 gene, were modified from those of Datta and colleagues (3) taking care to retain mismatches with the known K19 pseudogene (12) at the 3′ ends: sense primer 5′ GACTACAGCCACTACTACACGACC 3′ and antisense primer 5′ AGCCGCGACTTGATGTCCATGAGCC 3′. The PCR conditions were 94°C for 5 min followed by 45 cycles of 94°C for 1 min, 68°C for 1 min and 72°C for 1 min, with a final 7-min extension at 72°C.

A 20-μL aliquot of the PCR product was then electrophoresed on a 1.5% agarose gel, visualized by ethidium bromide staining, transferred by Southern blotting to a nylon filter (Hybond-N®+; Amersham International, Little Chalfont, Bucks, England, UK) and hybridized at 42°C with an internal oligonucleotide probe 5′ GATCTGCATCTCCAGGTCGGTCC 3′, which had been end-labeled with [γ-^{32}P]ATP (Bresatec, Adelaide, South Australia). After washing twice at 42°C in 2× standard saline citrate (SSC), 0.1% sodium dodecyl sulfate (SDS), autoradiography using Hyperfilm™ -MP (Amersham International) was carried out for 16–72 h.

Table 2. Efficacy of Labeling of Breast Cancer Cell Lines with Ber-EP4 as Assessed by Flow Cytometry

	Proportion of Cells Binding to Antibody		
	EMA	BC-2	Ber-EP4
MCF7	87.2%	75.4%	99.9%
T-47D	95.5%	71.0%	99.8%
MDA-MB453	12.0%	0%	100%

RESULTS

Selection of Antibody

The flow cytometry studies on the T-

47D, MCF7 and MB-453 breast cancer cell lines showed that the proportion of cells binding to Ber-EP4 was greater than the proportion binding to BC-2 or EMA (Table 2). The median fluorescence for Ber-EP4 was also greater (results not shown). Immunohistochemical studies of nine human breast cancer specimens showed consistent staining of all tumor cells by Ber-EP4.

Comparison of Cell Lysis Methods

A signal from approximately 50 cells could regularly be seen on ethidium bromide staining alone using either NP40 lysis or freeze-thaw lysis; although NP40 lysis gave consistently stronger bands (results not shown). Combining freeze-thawing with NP40 was no better than NP40 alone. Using heat lysis, a tenfold increase in cell number was required to observe bands on ethidium bromide staining.

Optimization of RT-PCR

Under conditions of low stringency (annealing temperature of 60°C), amplification of the 751-bp K19 band was seen sporadically from samples of genomic DNA (results not shown), and sporadic amplification of the same band was seen at RT-PCR on immunobead isolates from normal whole blood alone. Amplification from the same genomic DNA samples was abolished at a PCR annealing temperature of 68°C, and under these conditions, K19 amplification was only seen from one of 13 control normal peripheral blood samples.

RT-PCR, using an annealing temperature of 68°C and autoradiography could reliably detect a K19 signal from a single T-47D cell (Figure 1), without having to use nested PCR.

Immunobead Isolation of Carcinoma Cells

The RT-PCR assay, when applied to the immunobead-cell isolates and enhanced by the sensitivity of Southern blotting and autoradiography, could reliably display the expected 751-bp K19 band from 50 tumor cells in 10 mL of whole blood, with a limit of sensitivity of approximately 25 cells (Figure 2). As 10 mL of normal blood contains $4–9 \times 10^7$ white blood cells, the overall sensitivity was better than one breast cancer cell in one million white blood cells.

DISCUSSION

We have previously reported detection of colorectal cancer cells in peripheral blood by the immunobead PCR technique using mutations in codon 12 of the K-*ras* gene as the marker for the malignant cells (5,6). This approach has the disadvantage that circulating tumor cells can only be detected in patients whose primary tumor contains the K-*ras* codon 12 mutation. In the absence of a sufficiently frequent gene mutation in breast cancers, we have directed our attention towards mRNA-based markers that have the advantage of application to virtually all carcinomas.

The technique of immunobead RT-PCR relies on the preliminary isolation

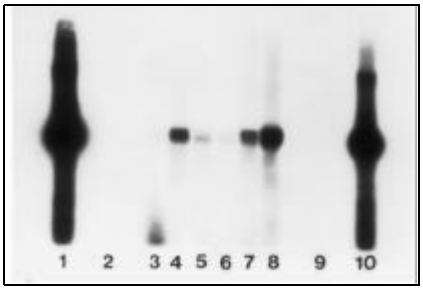

Figure 1. Single cell RT-PCR and autoradiography for K19 using T-47D breast cancer cells. Lane 1, PCR positive control (T-47D cDNA); lane 2, PCR negative control (no cDNA); lanes 3–6, single T-47D cells; lane 7, two T-47D cells; lane 8, five T-47D cells; lane 9, RT negative control (no cells); lane 10, RT positive control (100 T-47D cells).

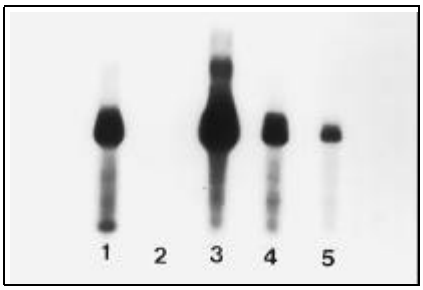

Figure 2. Sensitivity of immunobead RT-PCR for serial dilutions of T-47D cells. The cells were serially diluted in 10 mL whole blood, followed by immunobead RT-PCR. Lane 1, positive control; lane 2, negative control (no cells); lane 3, 500 T-47D cells; lane 4, 50 T-47D cells; lane 5, 25 T-47D cells.

of tumor cells from body fluids followed by amplification of one or more mRNA markers by RT-PCR. The monoclonal antibody Ber-EP4 binds to two glycoproteins that are specific to epithelial cells and that are retained in carcinomas (9). The ability of Ber-EP4 to bind to colon cancer cell lines and tissue samples has been demonstrated (5,9,16), and its applicability to breast cancer is confirmed here.

Since as few as one cancer cell may be bound by the immunobeads, the RT-PCR assay to detect the bound cells must be as sensitive as possible. The cell lysis technique reported here allows reliable amplification of a mRNA signal from a single cell, in this case, K19 expression from cells of a breast cancer cell line.The cell lysis method for reverse transcription has been demonstrated by us to be most efficient using NP40 at room temperature. At levels of 500 and 50 T-47D cells, NP40 lysis gave superior results to heating (at 94°C) or freeze-thawing. The poor results with the heat-lysis method may indicate that this method is relatively inefficient at lysing cells or may have been due to RNA degradation before the addition of RNasin.

Early optimism for K19 as a marker for carcinoma cells (3,15) has been countered by recent evidence that K19 transcripts can be sporadically amplified from the blood of healthy controls (2,8). Both papers have discussed potential problems from amplification of the K19 pseudogene. Since our technique is based on cell lysis and does not eliminate genomic DNA from the RT-PCR, it must be shown by the use of appropriate controls that the signal we observe is solely because of the presence of the mRNA of our chosen marker. We have confirmed the results of Datta et al. (3) that K19 pseudogene amplification can be avoided by a combination of appropriate primer design and choice of annealing temperature.

The recent findings of lack of specificity of K19 RT-PCR for detection of circulating carcinoma cells highlights the need for strict controls in the application of any RNA-based marker to cell detection by PCR. The prior tumor cell enrichment by the immunobead isolation in our technique leads to an RNA target that is much smaller, but with a far greater proportion of tumor-derived RNA, than methods based on total RNA extraction from whole blood (1–3,10,11,13,14). Immunobead isolation of the tumor cells is chiefly responsible for the sensitivity of the immunobead RT-PCR technique and avoids the need for nested PCR, thereby decreasing the risk of amplification of illegitimate mRNA transcripts.

This method has been optimized using breast cancer cell lines, but is equally applicable to the study of other carcinomas such as those of the colon and lung. The specificity of the immunobead RT-PCR technique depends upon the marker used for PCR. In this case, cytokeratin 19 has been used, but other RNA-based markers can be equally applied.

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Deoxyribonuclease Treatment Improves the Homogeneity of Single-Stranded DNA Preparations

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ABSTRACT

The isolation of single-stranded (ss) phagemid DNA using standard protocols often results in impure preparations, which contain undesirable quantities of chromosomal and/or double-stranded (ds) phagemid DNA. Here we report a simple and efficient method for elimination of virtually all dsDNA by incubation of phagemid viral particles with deoxyribonuclease I. In addition to analyzing the ratio of linear-to-circular topological forms of ssDNA after deoxyribonuclease I treatment, we verified that no decrease in transformation efficiency occurred and demonstrated that ssDNA molecules covered by capsid proteins remained intact following such treatment.

INTRODUCTION

Single-stranded DNA (ssDNA) molecules of filamentous phages or phagemid vectors are widely used in molecular biology experiments. Since it was first described, helper phage mediated single-stranded phagemid DNA isolation (6) has become a routine tool used in the preparation of template DNA for site-specific mutagenesis and sequencing.

The ssDNAs isolated by standard techniques always contain some amount of chromosomal and/or double-stranded phagemid DNA (dsDNA). The presence of dsDNA may cause mispriming events that result in ambi-