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Advances in Brief

Immunobead-PCR: A Technique for the Detection of Circulating Tumor Cells Using Immunomagnetic Beads and the Polymerase Chain Reaction

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Abstract

The presence of tumor cells in the circulation may predict disease recurrence and metastases. We have developed a sensitive technique for the detection of carcinoma cells in blood, using immunomagnetic beads to enrich for epithelial cells and the polymerase chain reaction to identify a tumor marker. The colon carcinoma cell line SW480, homozygous for a K-*ras* codon 12 mutation, was used to establish optimal conditions. The SW480 cells were serially diluted in normal blood and incubated with immunomagnetic beads labeled with a monoclonal antibody specific for epithelial cells. Cells bound to the beads were retrieved using a magnetic field and the presence of K-*ras* codon 12 mutations determined by a polymerase chain reaction based analysis. SW480 cells could be detected in dilutions up to 1 SW480 cell/10^5 leukocytes in whole blood.

Introduction

Despite recent advances in cancer treatment, recurrent and metastatic carcinomas, such as colorectal, lung, breast, and prostate, continue to pose a major problem in clinical management. These diseases are responsible for over half the cancer deaths in western countries (1, 2). Adjuvant therapy is being used to minimize the risk of late recurrences and strong evidence exists for its benefit in both breast (3, 4) and colorectal carcinoma (5). The significance of the presence of tumor cells in the circulation is as yet undetermined. The process of metastasis involves multiple host-tumor interactions and it is thought that less than 0.01% of circulating tumor cells are successful in establishing metastatic colonies (reviewed in Ref. 6). However, the ability to detect very small numbers of circulating tumor cells is likely to provide the clinician with an important predictive tool with respect to recurrence and metastases and to result in a more appropriate selection of patients for adjuvant therapy.

In designing a sensitive technique for the detection of carcinoma cells in blood, we chose colorectal cancer as our model. One of the earliest genetic changes in the progression of colorectal tumors is an activating point mutation in codon 12 of the K-*ras* gene, which is readily detected using the PCR.[2] Codon 12 mutations have been found in 50% of colorectal carcinomas (7–9), 80% of pancreatic carcinomas (10), and 30% of lung carcinomas (11). Detection of such a marker in the tumor specimen provides a means whereby tumor cells bearing that marker can be identified in blood.

To increase the sensitivity of detection of tumor cells, immunomagnetic beads, labeled with an epithelium-specific monoclonal antibody, were used to isolate colorectal tumor cells from blood. This technique is similar in concept to one that has been successful in isolating fetal trophoblast cells from maternal blood (12). PCR was then performed directly on the cell-bead isolates, using primers that introduce restriction enzyme sites into the PCR products. A non-radioactive RFLP analysis then allowed identification of mutant and normal K-*ras* sequences (13).

Materials and Methods

Colorectal Carcinoma Cell Lines

SW480 (14) was used as the source of target cells in dilution experiments and PCR sensitivity assays. LIM-1863, LIM-2463, LIM-2405, LIM-1215, LIM-2412 (15), and SW480 were used to assess reactivity of the monoclonal antibodies. The cell lines were kindly provided by Dr. E. J. Stanbridge, University of California at Irvine (SW480), and by Dr. R. Whitehead, Ludwig Institute for Cancer Research, Melbourne, Australia (LIM). The cells were maintained in culture in RPMI 1640 (Gibco BRL, Grand Island, NY) with 5% fetal calf serum.

Monoclonal Antibodies

Dako-Ber-EP4 (16), Dako-CEA, A5B7 (17), and Dako-EMA (Dakopatts, Gestrop, Denmark) were used in labeling experiments on cell lines at concentrations of 20 ng–4.0 μg/1 × 10^6 cells and the results were assessed by flow cytometry on a Coulter Epics Profile II.

Antibody Labeling of Immunomagnetic Beads

One million immunomagnetic beads (Dynabeads M-450, supplied covalently coated with sheep anti-mouse IgG, Dynal, Oslo, Norway) were incubated with Ber-EP4 at concentrations of 50 ng–4.0 μg/mg of beads according to the manufacturer's recommendations. The results were assessed by flow cytometry.

Immunomagnetic Bead and Tumor Cell Binding

Varying concentrations of Ber-EP4 labeled Dynabeads were incubated with SW480 cells. Bead:cell ratios of 1:5 to 1:30 were assessed by light microscopy.

Recovery Experiments

SW480 cells were serially diluted 10-fold into 10-ml aliquots of whole blood diluted to give SW480:white cell ratios from 1:10^3 to 1:10^6. (The blood used in these experiments was obtained from polycythemic patients undergoing large volume venesection). Labeled Dynabeads were added in a minimum ratio of 20:1 (2 × 10^6) to each tube and incubated with mixing at room temperature or at 4°C for 1 h. Isolation of cells bound to beads was achieved by taping the tubes to an array of cobalt-samarium disc magnets. The beads and bound cells were held against the side of the tube while the unbound cells were washed away (3 times in Hanks' balanced salt solution). The bead/cell aggregates were then pelleted into a PCR tube, resuspended in 10 μl of H_2O, and stored at -20°C until assayed.

PCR-RFLP Analysis

PCR Primers. A primer-mediated RFLP assay was used to identify K-*ras* codon 12 mutations. The primers were those of Jiang *et al.* (13); KRAS5',

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[2] The abbreviations used are: PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; CEA, carcinoembryonic antigen; EMA, epithelial membrane antigen.

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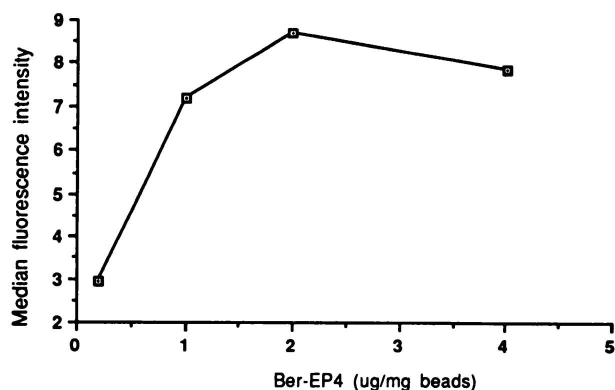

Fig. 1. Dynabeads labeled with Ber-Ep4; median fluorescence intensity at concentrations of 0.2, 1.0, 2.0, and 4.0 μg Ber-Ep4/mg Dynabeads.

5'ACTGAATATAAACTTGTGGTAGTTGGACCT3', and KRAS3', 5'TCAAAGAATGGTCCTGGACC3'. The mismatch (bold) in the 5' primer introduces a *Bst*N1 cutting site when normal K-*ras* sequences are amplified, but not when there is a mutation in the first or second position of codon 12 of K-*ras*. The mismatch (bold) in the 3' primer introduces a *Bst*N1 site into both normal and mutant products to serve as a control for enzyme digestion.

PCR. The tubes containing the bead/cell aggregates were heated at 100°C for 3 min, then 50 μl of the following reagents were added as a master mix: 10 mM Tris-HCl (pH 8.3); 1.5 mM $MgCl_2$; 50 mM KCl; 1 mg/ml gelatin; 200 μM concentrations, of each deoxynucleotide triphosphate (Boehringer Mannheim); 0.4 μM concentrations of each primer; 250 μM spermidine; 1 unit of Taq DNA polymerase (Amplitaq; Perkin-Elmer Cetus) and deionized H_2O. The tubes were heated at 94°C for 5 min and then subjected to 45 cycles of 94°C/30 s, 55°C/30 s and 72°C/30 s with a 7-min extension at 72°C. The PCR was optimized by varying the cycling parameters and the concentrations of primers, Mg^{2+}, and Taq polymerase.

Restriction Enzyme Digestion. PCR product (10 μl) was digested with 2 units of *Bst*N1 according to the manufacturer's recommendations (New England Biolabs, Beverly, MA). Ten μl of the digested products were electrophoresed on a 3% agarose or 10% polyacrylamide gel. The bands were photographed on a UV transilluminator after staining in ethidium bromide.

Sensitivity Assays. PCR was performed using SW480 DNA of known concentration, serially diluted from 1 μg to 1 pg.

Results

Using flow cytometry, the monoclonal antibody Ber-EP4 showed at least a 100-fold increase in median fluorescence compared to the same concentration (2 μg/10^6 cells) of CEA or EMA monoclonal antibodies for 5 of the 6 cell lines tested; the remaining cell line, LIM-2463, showed a 10-fold increase. All six cell lines were also >99.5% positive with Ber-EP4 in concentrations down to 100 ng/10^6 cells. On the other hand, the antibodies against CEA and EMA reacted with only a proportion of the cells, even at high antibody concentrations (2–4 μg/10^6 cells); CEA reacted with 3 of 5 cell lines (62, 65, and 72%) and EMA reacted with only 2 of the 6 cell lines (18%, 33%). Ber-EP4 was

Fig. 2. Photomicrographs of SW480 cells in blood incubated with Dynabeads in varying ratios of beads to tumor cells: *A*, 5:1; *B*, 10:1; *C*, 20:1; *D*, 30:1.

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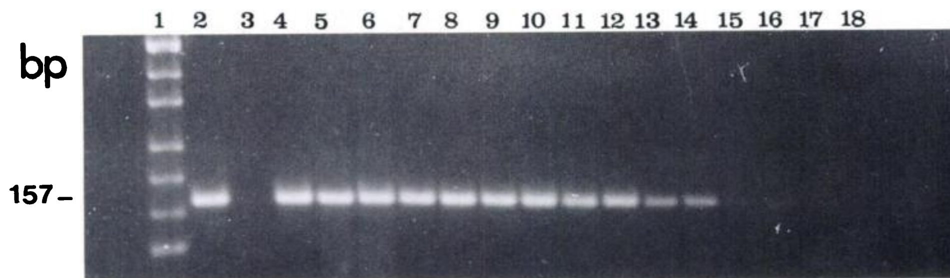

Fig. 3. PCR sensitivity using serial dilutions of SW480 DNA. *Lane 1*, pUC19/Hpa2 marker (bands of 501/489, 404, 331, 242, 190, 147, and 111/110); *Lane 2*, amplification control; *Lane 3*, negative (no DNA) control; *Lane 4*, SW480 control; *Lanes 5–18*, decreasing amounts of SW480 DNA; 1 μg (*Lanes 5, 6*), 100 ng (*Lanes 7, 8*), 10 ng (*Lanes 9, 10*), 1 ng (*Lanes 11, 12*), 100 pg (*Lanes 13, 14*), 10 pg (*Lanes 15, 16*), 1 pg (*Lanes 17, 18*). *bp*, base pairs.

therefore selected to label the Dynabeads used in the experiments to isolate SW480 cells "seeded" into normal blood.

The amount of Ber-EP4 required for labeling the immunomagnetic beads was assessed by flow cytometry. Saturation labeling, measured by median fluorescence, was obtained at a concentration of 2 μg Ber-EP4/mg beads (Fig. 1).

A ratio of 20:1 gave optimal binding of antibody-labeled beads to SW480 cells (Fig. 2) and was used in subsequent experiments. No binding of beads to hematopoietic cells was seen. Incubating the SW480/blood dilutions with labeled beads at room temperature compared to 4°C resulted in a 10-fold increase in sensitivity of detection. The presence of beads in the PCR mix did not affect amplification (data not shown).

PCR analysis of SW480 DNA serially diluted in H_2O showed that the 143-base pair mutant band could be consistently detected on ethidium bromide-stained gels following amplification of 10 pg but not 1 pg (<1 cell) of DNA (Fig. 3).

PCR-RFLP analysis proved to be very sensitive following immunobead isolation of tumor cells (Fig. 4). After digestion of PCR products with *Bst*N1, the 143-base pair mutant band could be detected on ethidium bromide-stained gels down to the $1:10^5$ dilution of SW480 cells in normal blood. Bands at 114 base pairs, representing the normal sequence, show that there is comparatively low contamination with normal cells.

Discussion

We have developed a sensitive technique for the detection of tumor cells in blood. This technique is dependent on antibodies which can reliably bind to the target cells. We have tested three monoclonal antibodies for their ability to label surface epitopes on a panel of six colon carcinoma cell lines. Ber-EP4 was the best of the three antibodies as assessed by flow cytometry. Ber-EP4 reacts with two glycopeptides with molecular weights of 34,000 and 39,000 present on the surface and in the cytoplasm of all epithelial cells except the superficial layers of squamous epithelia, hepatocytes, and parietal cells (16). Ber-EP4 does not label hematopoietic cells (data not shown).

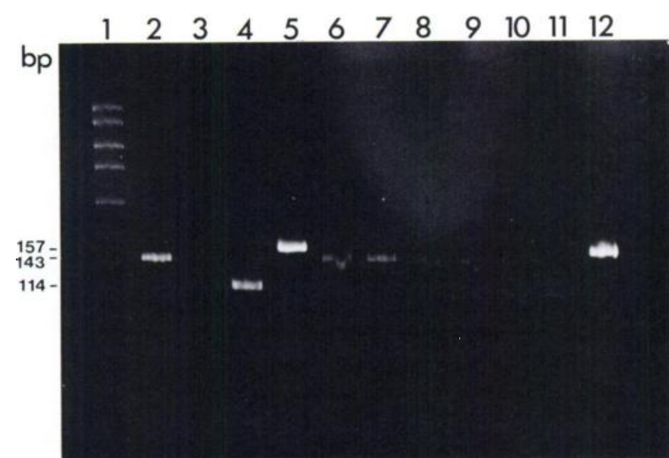

Fig. 4. Detection of mutant K-*ras* sequences after immunobead-PCR and *Bst*N1 digestion. *Lane 1*, puc19/Hpa2 markers: *Lane 2*, SW480 [143 base pairs (bp)], *Lane 3*, negative (no DNA) control; *Lane 4*, normal control (114 base pairs), *Lane 5*, undigested control (157 base pairs), *Lanes 6–11*, dilutions of SW480 into whole blood relative to the number of leukocytes, $1/10^4$ (*Lanes 6* and *7*), $1/10^5$ (*Lanes 8* and *9*), $1/10^6$ (*Lanes 10* and *11*); *Lane 12*, undigested control (157 base pairs).

The immunobead-PCR technique reported here is many times more sensitive than that reported by Jiang *et al.* (13), who were able to detect the K-*ras* mutant band at up to a 1:16 dilution. Our more than 1000-fold increase in sensitivity is due to the prior enrichment of tumor cells with immunobeads. Modifications of this method may give further improvements in sensitivity, *e.g.*, by the prior separation of nucleated cells from blood, since the RBC may sterically hinder the interaction of antibody-coated beads and target cells.

Recently, Kahn *et al.* (18) described a modification of the PCR technique of Jiang *et al.* (13) allowing significantly better detection ($1/10^4$) of mutant K-*ras* sequences. Their technique is still 10-fold less sensitive than the immunobead-PCR method presented here. Use of their method in our system is unlikely to increase our sensitivity, since we have limited contamination with normal cells (Fig. 4).

This technique can be used to begin to address questions regarding the dynamics of exfoliation of tumor cells into the bloodstream and their removal from the circulation by extravasation (6). It can also be used to examine the release of potentially metastatic tumor cells in the blood of patients undergoing surgical resection of their tumor.

There is evidence that the presence of tumor cells in the circulation is a prognostic indicator of recurrence and metastatic disease (19, 20). Thus, the detection of circulating tumor cells may result in an earlier and more appropriate selection of patients for further therapy.

This technique could be applied to the detection of any metastasizing cell of nonhematopoietic origin, providing that an appropriate antibody and tumor-specific tumor marker are available.

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