

The Prediction of Breast Cancer Contamination in Stem Cell Products by Immunocytochemical Analysis of Premobilized Peripheral Blood

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ABSTRACT

One of the end points of the collection of peripheral blood stem cells (PBSCs) before marrow-ablative chemotherapy and autologous transplantation is tumor-free product. One way to obtain a tumor-free graft is to perform purging. However, because purging is an expensive procedure, purging of all grafts would not be cost-effective. To determine if one could predict the contamination of a stem cell graft, we evaluated premobilized peripheral blood (PB) for the presence of tumor cells. Using a routine immunocytochemical (ICC) assay (sensitivity 1/1,000,000), tumor cells were detected before mobilization in PB of 32 (5%) of 635 breast cancer patients. Paired premobilized PB specimens and samples from PBSC products were available from 460 patients. From these paired samples, 25 PB (5.4%) and 100 PBSC (21.7%) samples were positive for tumor cells. Breast cancer cells were detected in 24 of 25 PBSC samples (96%) when the premobilized PB sample contained tumor cells. Due to the low positive rate of PB samples, a number of PBSC products positive for tumor cells went unpredicted. Therefore, it was necessary to develop a tumor-enriched ICC (EICC) assay that could detect routinely 1/10,000,000 tumor cells. In seeding experiments, CAMA cells were seeded into aliquots of PBSCs from normal donors at concentrations of 1/1,000,000, 1/5,000,000, 1/10,000,000, 1/20,000,000, and 1/50,000,000. Overall tumor cell recovery in the positive fractions of the various seeded samples ranged between 56% and 97%. This EICC system was then used to enrich tumor cells from PB of breast cancer patients. For 107 PB samples of early stage (I–III) and 42 of stage IV patients, EICC-positive cells were found in 23 (21%) and 25 (59.5%) patients, respectively. The EICC assay now permits testing of PB for residual cancer cells in a larger percentage of breast cancer patients. The use of this assay in premobilized PB may permit the early identification of subset of patients with an extremely high probability of having breast cancer cells in their PBSC products, who may require purging of their products.

INTRODUCTION

High-dose chemotherapy with autologous stem cell transplant may be an effective treatment for patients with advanced breast cancer.¹ The major reason for peripheral blood stem cell treatment failure is relapse of disease, which may in part be a result of reinfusion of tumor cells in the graft.² The presence of tumor cells in the reinfused grafts of some breast cancer patients is well established.³⁻⁵ Circulating breast cancer cells have been found to increase as a result of the type of mobilization regimens used and with the number of PBSC collections.^{6,7} In addition, these contaminating cells have been shown to be viable and are capable in vitro clonogenic growth.^{3,4} Gene-marking studies of patients with chronic myeloid leukemia, lymphoma, and neuroblastoma have shown that tumor cells in the graft contribute to relapse in patients.⁸⁻¹⁰ Finally, the presence of tumor cells in stem cell products has been correlated with a poor posttransplant clinical outcome.¹¹⁻¹⁴

One way to get a tumor-free PBSC product is to purge the graft. A variety of approaches for tumor purging have been characterized. These include immunomagnetic cell depletion via positive or negative selection, pharmacological purging (such as 4-hydroperoxycyclophosphamide), immunotoxins, and density gradient separation.¹⁵⁻¹⁸ Purging of all PBSC products may be prohibitively expensive, particularly when the contamination is only 10%–25%. Therefore, if we can predict which PBSCs are positive before harvesting, we could purge only a subset of PBSC products and provide better cost efficiency.

The aim of this study was to develop a premobilized assay that can predict the presence of tumor cells in PBSC products of transplant patients. We have been using an alkaline phosphatase (AP)-based ICC assay with a routine sensitivity of 1/1,000,000. Using this assay, premobilized PB cells of patients were evaluated for contaminating tumor cells and correlated with tumor cell contamination in their PBSC products. When the PB was positive, the assay had high predictive power to identify patients with breast cancer cells in PBSC infusions. However, a major problem was that a number of ICC-negative premobilized PB samples did not predict for a negative PBSC harvest. This may be due to inadequate sensitivity of the ICC assay for the evaluation of blood.

To identify breast cancer cells that are present in low numbers in premobilized PB, we developed an ultrasensitive tumor-enriched immunocytochemical micrometastatic assay that has a sensitivity of 1/10,000,000. This system uses the Miltenyi magnetic cell sorting (MACS) immunomagnetic selection device using colloidal superparamagnetic microbeads conjugated with anti-epithelial (HEA-125) monoclonal antibody (mAb) to enrich tumor cells before ICC analysis.

MATERIALS AND METHODS

Cell Lines

The breast cancer cell line CAMA was maintained in 15% fetal bovine serum (FBS) (Gibco/BRL, Life Technologies, Rockville, MD) and Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) with 2 μ M L-glutamine and 100 U/mL penicillin-streptomycin.

Monoclonal Antibodies

For ICC analysis, antibodies previously described were used.⁴ For EICC, SB-3/TFS-2 anti-cytokeratin mAb cocktail was used. TFS2 mAb (IgG2b; Biodesign International, Kennebunkport, ME) recognizes a 39-kDa carcinoma-specific surface antigen, and the SB-3 mAb (MeDica, Carlsbad, CA) recognizes an epitope on cytokeratin 8 (CK8) and CK18.

Processing and Immunostaining

Aliquots of PBSCs (at least 2×10^7 cells) were shipped at room temperature to IMPATH/BIS for ICC analysis. Mononuclear cell fractions were isolated by density gradient centrifugation using Ficoll-Hypaque (Pharmacia & Upjohn, Bridgewater, NJ) and washed twice in Leibovitz L-15 medium (Gibco/BRL) supplemented with 10% FBS. The mononuclear fraction was collected, and cells were spun onto slides for immunostaining. Slides were fixed in paraformaldehyde/methanol (2:1) or 4% paraformaldehyde fixative, washed thoroughly in Dulbecco's modified phosphate-buffered saline (PBS) (Gibco/BRL), and placed on an automated immunostainer (TechMate; Ventana, Tucson, AZ). AP immunostaining was then performed per manufacturer's instructions as previously described.¹² Briefly, slides were incubated in the following order: blocking solution, primary antibody cocktail, secondary antibody, AP complex, chromogen, and hematoxylin. Buffer washes were performed between each step. Positive control slides consisted of CAMA cells seeded into normal PB or bone marrow and immunostained as above. Negative control slides were the patient's specimen immunostained with normal mouse serum at the same concentration as used for the anti-breast cancer antibodies.

Quantitation of Tumor Cell Concentration

Slides were evaluated on a blinded basis, and the total number of tumor cells, identified by microscopic evaluation, was recorded. In addition to displaying immunostaining, cells had to exhibit morphology consistent with malignant

phenotype to score as breast cancer cells. For standard ICC, tumor cell frequency was calculated as the quotient of total number of tumor cells detected divided by total number of cells on the slides. For EICC, tumor frequency was derived by taking the total number of cells detected and dividing by the number of cells used for enrichment.

Generation of Seeded Tumor Cells

CAMA cultured breast cancer cells were removed from tissue culture flasks, washed twice, and placed in L-15/FBS medium at a concentration of 1×10^6 cells/mL.

The hematopoietic cell fractions from normal donors were divided into six 2×10^8 cell fractions. Tumor cells were spiked into these fractions at concentrations of 0, 1/1,000,000, 1/5,000,000, 1/10,000,000, 1/20,000,000, and 1/50,000,000. Enrichment for tumor cells using HEA-125 mAb-conjugated beads was performed on all cell aliquots. Cytopreparations from the nonenriched and enriched material were made for all aliquots.

EICC Assay

Figure 1 shows a diagram of enrichment of disseminated carcinoma cells using the HEA microbeads and MACS technology. The mononuclear cell fraction was isolated by Ficoll-Hypaque separation, washed twice in Leibovitz L-15 medium supplemented with 10% FBS, and placed in L-15/FBS at a concentration of 1×10^8 cells/mL. Cells were then subjected to immunomagnetic cell selection using an anti-HEA-125 mAb magnetic microbead conjugate

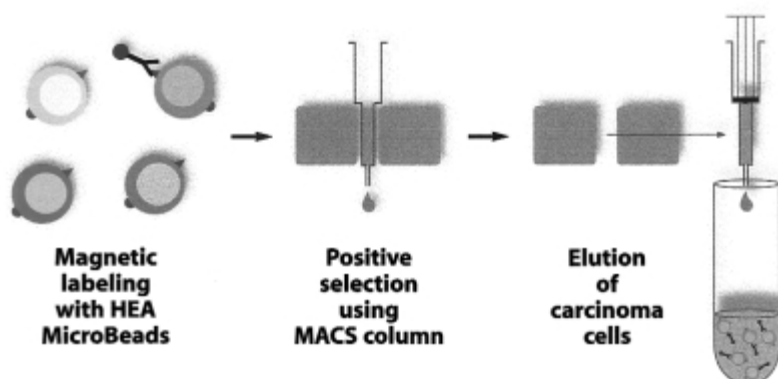


Figure 1. Schematic representation for enrichment of disseminated carcinoma cells using HEA-125 microbeads and magnetic cell sorting (MACS) technology.

(Miltenyi Biotec, Auburn, CA). The HEA-125 antigen is an epithelial-specific surface glycoprotein, widely expressed by human carcinoma.¹⁹ The system and magnetic cell separation columns were used according to manufacturer's instructions. Briefly, mononuclear cells from PB or PBSC were placed in PBS containing 0.2% sodium citrate and washed twice at 1000 rpm for 10 minutes each. After washing, cells were incubated with a blocking reagent and HEA-125 antibody-conjugated microbeads at 4°C for 30 minutes. The cells were washed twice with PBS and sodium citrate (0.2%) to remove unbound beads and placed in a separation column along with a MiniMACS magnet for 2 minutes at room temperature to bind cells. Bound cells were removed by gentle flushing of the column with 1 mL buffer using a plunger into fresh tube. All bead:cell conjugates recovered from the magnet were used in cytopreparations and immunostained. Two to 4 cytopreparations were made with the Shandon cytospin and stored for immunostaining at 4°C. Cytopreparations were fixed in 4% paraformaldehyde fixative, washed thoroughly in Dulbecco's modified PBS with 1% Triton X, and placed on an automated immunostainer. AP immunostaining was performed per protocol.

RESULTS

Detection of Tumor Cells in Premobilized Blood Specimens

An ICC-based assay with a routine sensitivity of 1/1,000,000 was used to evaluate premobilized PB cells of stage II (high risk), stage III, and stage IV breast cancer patients for contaminating tumor cells and to correlate with tumor cell contamination in their PBSC products.

PB before mobilization was available from 635 breast cancer patients. ICC was positive in 32 (5.0%). Paired premobilized PB specimens and PBSC products were available from 460 patients. From these paired samples, 25 PB (5.4%) and 100 PBSC (21.7%) specimens were positive for tumor cells. The range of tumor cells detected was 1/100,000 to 1/6,000,000, median 1/2,000,000. The results of the paired samples are summarized in Table 1. Breast cancer cells were detected in 24 of 25 PBSC products (96%) when the premobilized PB contained tumor cells. However, in 77 of 435 patients, the premobilized PB was ICC negative, yet the PBSC had tumor cells present (false-negative rate of 17.7%). Although a positive ICC of premobilized PB was highly predictive of a positive PBSC, there were a number of false-negative cases. This false-negative rate was probably due to the presence of tumor cells in PB at concentrations less than 1/1,000,000. Thus, we developed a tumor-enriched assay.

Table 1. Tumor Cell Contamination Using Immunocytochemical (ICC) Assay in Paired Peripheral Premobilized Blood and Peripheral Blood Progenitor Cells (PBSCs) of Breast Cancer Patients*

| | | | | |
|--------------------|-----|----|----|---|
| Number of patients | 358 | 77 | 24 | 1 |
| Peripheral blood | – | – | + | + |
| PBSCs | – | + | + | – |

*–, no tumor cells were found in ICC analysis; +, tumor cells were detected.

EICC Tumor Cell Seeding Experiments

The efficacy of the tumor enrichment assay was evaluated in a model system in which cultured CAMA breast cancer cells were seeded into PBSC products of normal donors. In initial studies, EICC analysis of PBSCs seeded with CAMA cells at a concentration of 1/1,000,000 was evaluated after enrichment with HEA-125 magnetic beads. Initial recovery studies of CAMA cells from PBSCs demonstrated an average recovery of 74% ($n = 16$, 52%–96%). No tumor cells were detected in any of the negative fractions following enrichment. In the next set of experiments, CAMA cells were seeded into aliquots of PBSCs from normal donors at concentrations of 1/1,000,000 (200 cells), 1/5,000,000 (40 cells), 1/10,000,000 (20 cells), 1/20,000,000 (10 cells), and 1/50,000,000 (4 cells). Samples of 2×10^8 cells were seeded and tested for tumor using the above enrichment system.

The results from the tumor seeding experiments showed that using the Miltenyi enrichment system, tumor cells were detected in all seeded products, even when seeded with as low as 1/50,000,000 (4 cells). Overall recovery in the various seeded samples in 4 independent experiments ranged between 56.2% and 97.5%. Mean tumor cell recovery was 97.2% (range, 37%–119%), 74.2% (14%–167%), 62% (30%–120%), 95% (10%–200), and 56.2% (0%–125%) of PBSC samples seeded at 1/1,000,000, 1/5,000,000, 1/10,000,000, 1/20,000,000, and 1/50,000,000, respectively.

The specificity of the enrichment assay was assessed using nonseeded PBSCs or PB from 50 normal donors or from patients with hematologic malignancies. None of these normal donor specimens tested positive.

Patient Specimens

The standard ICC and EICC tumor cell detection system was then tested and compared on PBSCs taken from breast cancer patients with various disease stages, all candidates for PBSC transplantation. The results are summarized in Table 2. Using standard ICC, contaminating tumor cells were found in 33%, 28.6%, and

16.6% compared with EICC, where tumor cell contamination was evident in 58%, 42.8%, and in 58% of patients' specimens for stage II, III, and IV, respectively. Tumor cell concentration ranged from 1/1,500,000 to 1/25,000,000. When the results of all 31 patients were combined, only 26% of patient specimens were found to be positive using the standard ICC assay vs. 55% using the EICC assay ($P=.002$).

The ICC and EICC results were compared on premobilized PB samples of breast cancer patients. Of 635 premobilized PB samples of breast cancer patients using standard ICC, the positive rate was 32 (5.0%), and in a different but comparable group of patients, the EICC positive rate was 25 of 42 (59.5%). Tumor concentration for these samples ranged from 6/10,000,000 to 2/100,000,000.

The power of the EICC assay was also tested on PB of early-stage patients with epithelial malignancies. There were 40 patients with breast cancer, 43 with prostate cancer, 15 with gastrointestinal cancer, and 9 with lung cancer. Of the 107 early-stage patients, 23 (21.0%) were positive for tumor cells in the PB. When circulating tumor cells were evaluated in PB of stage IV patients, a ~45% positive rate was found.

DISCUSSION

One of the end points of PBSCT is obtaining a tumor-free product. One way to get a tumor-free PBSC product is to use methods that would reduce the number of tumor cells in the graft, including alternative mobilization regimens and purging of the PBSC product.¹⁶⁻¹⁸

One promising method for purging is the indirect tumor reduction that occurs with positive selection of CD34⁺ cells from PBSC products.²⁰ Studies have demonstrated that ~1-2 logs of breast cancer tumor cells can be removed using this process.²¹⁻²³ Because this is an expensive procedure, however, the use of CD34 selection must be questioned when the PBSC is negative for tumor cells. Therefore, being able to predict which patient will have positive PBSCs could make CD34 collection more cost-effective.

Table 2. Comparison Between Standard Immunocytochemical (ICC) and Tumor-Enriched ICC (EICC) Results Using Peripheral Blood Stem Cell Specimens Taken From Breast Cancer Patients at Various Stages of Disease*

| <i>Stage</i> | <i>Specimens</i> | <i>ICC</i> | <i>EICC</i> |
|-------------------------|------------------|------------|-------------|
| II | 12 | 4 (33.3) | 7 (58.3) |
| III | 7 | 2 (28.6) | 3 (42.8) |
| IV | 12 | 2 (16.6) | 7 (58.3) |
| Acute lymphoid leukemia | 31 | 8 (25.8) | 17 (54.8) |

* Data are n or n (%). $P=.002$.

In this study, we evaluated the power of premobilized ICC PB specimens for predicting PBSC tumor cell contamination. We hypothesized that if this assay proves to have predictive value, it would be possible to determine which PBSC products are positive before harvesting. The assay could permit transplant physicians to purge only a subset of PBSC products, rather than all products.

The standard ICC assay in this study, with a sensitivity of 1/1,000,000, was thus used to evaluate tumor cell contamination in premobilized PB and PBSC products. In paired samples, breast cancer cells were detected in 24 of 25 PBSC products (96%) when the premobilized PB sample contained tumor cells. However, in 77 of 435 patients, the premobilized PB ICC was negative, yet PBSCs had tumor cells present (a false-negative rate of 17.7%).

To improve the predictive power of this analysis, we developed an ultrasensitive tumor-enriched assay with a sensitivity of 1/10,000,000. For EICC seeding experiments, a high tumor cell recovery with an overall mean tumor cell recovery between 56.2% and 97.5% was found. Moreover, tumor cell recovery was adequate even when tumor cell seeding concentrations were as low as 1/20,000,000 and 1/50,000,000.

The superior detection power of EICC was proven on PBSC and PB samples taken from cancer patients. For patients with breast cancer, the standard ICC assay was positive in 32 of 635 (5.0%), whereas the EICC assay was positive in 25 of 42 (59.5%). For patients with a variety of early-stage cancers, the EICC assay was positive in 23 of 107 (21%).

The breast cancer ultrasensitive EICC method described in this study showed a significant increase in sensitivity of tumor detection in premobilized PB as well as in PBSC products compared with standard ICC. Based on the predictive power of ICC-analyzed premobilized PB, we anticipate that the EICC analysis will greatly reduce the false-negative rate. This EICC approach could be used to predict the presence of tumor cells in PBSC products before mobilization. The information obtained can then be applied to determine the need for purging subsets of grafts rather than all the grafts. In addition, the EICC assay has potential to be used in future investigations to study the relationship between premobilized PB contamination and patient outcomes. Studies toward this end are currently being conducted in our laboratory.

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