Breast Cancer Cells Are Effectively Purged from Peripheral Blood Progenitor Cells with an Immunomagnetic Technique


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ABSTRACT

Peripheral blood progenitor cells (PBPCs) are being used increasingly to provide hematopoietic support after intensive chemotherapy. However, many investigators have detected tumor cells contaminating PBPC collections. Methods that eliminate the tumor cells and spare the normal hematopoietic progenitor cells may improve the number of long-term, disease-free survivors after intensive chemotherapy. We developed an effective method using anti-breast cancer murine monoclonal antibodies (MoAbs) and immunomagnetic beads to eliminate a low percentage of breast cancer cells from PBPCs. We identified optimal anti-breast cancer MoAbs that react with membrane glycoproteins and conditions for selective removal of tumor cells. Using three anti-breast cancer MoAbs (260F9, 317G5, and 520C9) at 0.8 μg/ml, a cell concentration of 2 x 10^8 cells/ml and a bead:total cell ratio of 0.75 beads:1 cell, we eliminated 3.3–4.8 (mean, 4.1) logs of tumor cells consistently from a model system with 1% breast cancer cells and 99% normal PBPCs. Similar levels of tumor cell elimination were obtained with three breast cancer cell lines. Colony-forming units were not affected adversely, with a mean recovery of 200% compared with the control. A clinical trial has begun that uses immunomagnetically purged, autologous bone marrow and PBPCs to support patients with metastatic breast cancer receiving high-dose chemotherapy.

INTRODUCTION

Dose intensification may be important in the treatment of many cancers. With most malignancies, the higher the dose of chemotherapy used, the higher the response rate and possibly a longer disease-free interval. High doses of myelotoxic agents require support with bone marrow or PBPCs to ensure hematopoietic recovery for these patients. High-dose chemotherapy and autologous bone marrow transplantation are used widely for the treatment of certain patients with acute leukemia, Hodgkin’s disease, non-Hodgkin’s lymphoma, neuroblastoma, and multiple myeloma (1). Recently, many solid-tumor patients, particularly breast cancer patients, have been treated with high-dose chemotherapy followed by autologous bone marrow transplantation in an effort to improve the long-term treatment results (2–5). The addition of PBPCs to autologous bone marrow decreases toxicity, mortality, and resource use (6, 7). Alternatively, PBPCs alone can support a person after high-dose chemotherapy adequately (8, 9).

A potential limitation of support with autologous bone marrow or PBPCs following high-dose chemotherapy is the possibility that contaminating tumor cells will be given back to the patient. Recent studies have shown that 17–28% of women with newly diagnosed breast cancer and no histological evidence of metastases in the bone marrow had tumor cells detected in their bone marrow by immunohistochemical methods (10, 11). Overt metastatic disease will develop in 50% of women with breast cancer within 5 years of initial diagnosis, and at first relapse, 13–57% will have bone marrow metastases (12–14). Recently, immunocytochemical methods have been used to study tumor contamination in PBPC collections. It was found that 9.8% of the PBPC collections from breast cancer patients contained tumor cells compared with 62.3% of the bone marrow samples studied (15).

A safe and effective purging technique may improve the number of long-term, disease-free survivors. Currently, several methods have been evaluated for this purpose, including cytotoxic drugs (16), lectins (17), immunotoxins (18–20), and MoAbs in combination with complement (21–23) or magnetic microspheres (24, 25). We chose to evaluate an immunomagnetic method using three murine anti-breast cancer MoAbs (260F9, 317G5, and 520C9) and magnetic microspheres coated with rabbit antimouse immunoglobulin to eliminate malignant breast cancer cells from human PBPCs. Using a model system, elimination of clonogenic breast cancer cells and preservation of normal hematopoietic progenitor cells were studied.

MATERIALS AND METHODS

Breast Cancer Cell Lines. The primary cell line, CAMA-1, was derived from a postmenopausal woman with an adenocarcinoma of the breast (26). Other breast cancer cell lines included SKBr3 (27) and BT-20 (28). Cells were grown in T-75 flasks as monolayers. CAMA-1 cells were maintained in DMEM with high glucose (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (GIBCO), 0.03% L-glutamine, 100 μg/ml penicillin, and 120 μg/ml streptomycin. SKBr3 and BT-20 cells were maintained in RPMI 1640 with the same supplements. Cells were detached with 0.25% trypsin-EDTA immediately before use.

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3 The abbreviations used are: PBPC, peripheral blood progenitor cell; MoAb, monoclonal antibody; CFU-GM, colony-forming unit-granulocyte-macrophage; BFU-E, burst-forming unit-erythrocyte.
Immunomagnetic Purging of PBPCs

PBPCs. Using protocols approved by the Duke University Medical Center Institutional Review Board, PBPCs were obtained from stage II breast cancer patients with more than three positive lymph nodes to permit evaluations of purging techniques with small numbers of cells. These PBPC collections were not pretested for the presence of tumor contamination. If present, these tumor cells would not be expected to grow in the limiting dilution, clonogenic assay, because only established cell lines have been shown to do this. PBPCs were collected from a healthy male volunteer to study scaleup of the technique. For the initial “small scale” studies, samples were washed twice with PBS at 1000 rpm for 10 min to remove citrate. The “large scale” PBPC collection was washed with 2 liters 0.9% sodium chloride solution on the Cobe 2991 blood cell processor before processing.

MoAbs. A panel of three murine MoAbs, reactive against epithelial cell surface determinants, three membrane glycoproteins, was used for immunoseparation of breast cancer cells from human PBPCs. Antibodies were chosen based on additive binding to breast cancer cell lines by indirect immunofluorescence and flow cytometry and also lacking reactivity to normal human bone marrow (29, 30). The panel included 260F9, 317G5, and 520C9, which recognize M, 55,000, 42,000, and 210,000 antigens, respectively.

Magnetospheres. M-450 Dynabeads coated with sheep antimonouse immunoglobulin were obtained from Dynal, Inc. (Lake Success, NY). The smooth-surfaced, monodispersed polystyrene beads contained approximately 20% magnetic Fe2O3 by weight and were 4.5 μm in diameter. A polyclonal sheep antimonouse immunoglobulin antibody was linked covalently to the particle surface after affinity purification and removal of antibodies reacting with human immunoglobulins by solid-phase absorption (31).

Immunoseparation with Magnetospheres and MoAbs. Experiments were performed using a mixture of 1 × 10^9-1 × 10^10 PBPCs and a 1% contamination with breast cancer cells using sterile 15-ml conical tubes for the small scale experiments. A large scale experiment was performed using 1.7 × 10^10 normal PBPCs in a volume of 150 ml, mixed with 1.7 × 10^8 CAMA-1 breast cancer cells. Cell suspensions were incubated with the three murine MoAbs for 1 h at 4°C. After excess unbound antibody was removed by washing, the magnetospheres were added for 1 h on ice with constant rotation. The treated cell suspension was placed on a hand-held samarium-cobalt magnet for 2 min. Nonadherent cells were decanted and assayed for residual clonogenic breast cancer cells in a limiting dilution assay and for PBPC recovery in a methylcellulose tissue culture assay. The bead:cell ratio and the concentrations of MoAbs and cells were changed to optimize the removal of clonogenic tumor cells and to minimize normal hematopoietic progenitor cell loss. The large scale cell suspension was placed on the Fenwal MaxSep magnetic cell separation system (Baxter, Deerfield, IL) to remove the adherent cells.

Limiting Dilution, Clonogenic Assay. A clonogenic assay was developed with breast cancer cell lines based on previous studies with human B- (22) and T-cell (32) lymphoma cell lines, which have been studied with many breast and small cell lung cancer cell lines (33, 34). A series of eight 5-fold dilutions were prepared from PBPC and tumor cell mixtures (1 × 10^6 total cells/ml) after immunoseparation. Six aliquots (100 μl) of each dilution were incubated in 96-well, flat-bottom microtiter plates, to which 100-μl aliquots of conditioned tissue culture medium had been added. Plates were then incubated for 14 days at 37°C in 5% CO2 and 95% humidified air. The growth of colonies was scored by visual inspection with an inverted-phase microscope, noting the number of wells with at least one colony containing ≥10 cells. The cells from clonogenic colonies were stained with an anticytokeratin MoAb, and, using an immunofluorescent assay, the clonogenic cells were found to be cytokeratin positive. The number of clonogenic units remaining was calculated with a Spearman-Karber estimator (35). The cloning efficiencies of the CAMA and SKBr3 cell lines are 93-99%; therefore, the limiting dilution cultures will detect one tumor cell more than 90% of the time. The log tumor cell removal is based on a comparison of the limiting dilution, clonogenic assays from a control plate, which was not purged, and the experimental plate, which was purged (33, 34). In addition, we repeated the experiments and changed the control; therefore, the control was purged in an identical fashion, except that the anti-breast cancer MoAbs were not included. The second control measures the nonspecific loss of clonogenic breast cancer cells as a result of the purging process.

Bone Marrow Progenitor Cell Assay. Terry Fox medium was used for the progenitor cell assays (Stem Cell Technologies; Terry Fox Laboratories, Vancouver, British Columbia, Canada). Cell suspensions were adjusted to 1 × 10^5 cells/ml from which 100 μl were plated in duplicate using Corning 12-well, flat-bottom plates (Corning Glass Co., Corning, NY) for a final plating concentration of 1 × 10^4 cells/well. Plates were incubated for 14 days in 5% CO2 with 95% humidified air at 37°C. CFU-GMs and BFU-Es were scored using an inverted-phase microscope.

RESULTS

Optimal Antibody Concentration for Immunoseparation. PBPCs were mixed with a 1% CAMA-1 tumor cell contamination. These suspensions were incubated with various concentrations of the three murine MoAbs for 1 h at 4°C. Between 3.5-4.4 logs of clonogenic breast cancer cells were eliminated, with maximal tumor cell elimination observed between 0.4 and 4 μg/ml of the tested range of each antibody (Fig. 1). This range is similar to that observed in previous studies, showing maximal antitumor activity between 0.2 and 20 μg/ml (14). Consequently, a MoAb concentration of 0.8 μg/ml was used for subsequent experiments. The median log tumor cell removals were 4.0 (range, 3.6-4.4) logs for both the 4- and 0.8-mg/ml concentrations and 3.7 (range, 3.5-3.9) logs for the 0.4 mg/ml concentration.

Optimal Magnetosphere Concentration for Immunoseparation. Using a concentration of 0.8 μg/ml each antibody, mixtures of breast cancer cell lines and PBPCs were incubated with different concentrations of magnetospheres prior to immunomagnetic separation. The ratios of immunomagnetic

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4 C. L. Tyer and J. J. Vredenburgh, unpublished data.
beads:total cells varied between 0.075 and 0.75 beads:cell. The recovery of CFU-GM was similar at the different bead:cell ratios. Our previous studies suggested that a further increase in the bead:cell ratio would affect the recovery of moval of 1% clonogenic CAMA-1 breast cancer cells mixed with mor cell removal was optimal with a bead:cell ratio of 0.75

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**Fig. 1** Effect of antibody concentration on the mean log tumor removal of 1% clonogenic CAMA-1 breast cancer cells mixed with PBPCs.

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**Optimal Working Cell Concentration for Immunoseparation.** PBPC collections can range from $1 \times 10^9$ to $1 \times 10^{11}$ cells/apheresis. We evaluated purging of tumor cells in the presence of different concentrations of PBPCs. Cell concentrations ranged from $5 \times 10^7$ to $1 \times 10^9$ cells/ml. Optimal tumor cell removal was obtained in the range of $2 \times 10^8$ to $3 \times 10^8$ total cells/ml. At these cell concentrations, the recovery of CFU-GMs was not affected (Table 1).

**Immunoseparation of Multiple Breast Cancer Cell Lines.** Similar experiments were performed with two additional breast cancer cell lines, SKBr3 and BT-20. Using immunomagnetic separation, 2.0–4.5 logs of tumor cells were eliminated. The extent of tumor elimination depended on the clonogenic efficiency of the breast cancer cell line (Fig. 2).

**Optimal Parameter Checks.** To confirm the utility of the treatment conditions, CAMA-1 breast cancer cells were mixed with samples of PBPCs at final concentrations of 1%, 0.1%, and 0.01% tumor cells/normal progenitor cells. From 4.3 to 4.8 logs of tumor cells could be eliminated using 0.8 μg/ml MoAb, with a bead:cell ratio of 0.75:1 and a working cell concentration of $2 \times 10^8$ cells/ml. When the negative control included the purging process without the anti-breast cancer MoAb, the log tumor cell removal of the purged sample minus the control was a median of 2.9 logs. Therefore, the nonspecific tumor cell loss was approximately 1.5 logs.

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**Scaleup of Techniques.** Following protocols approved by the Duke University Medical Center Institutional Review Board, a normal male donor was apheresed, and $1.7 \times 10^{10}$ PBPCs were obtained. CAMA-1 cells were mixed with PBPCs to reach a final concentration of 1% tumor cells. Mixtures were incubated with the MoAbs and immunomagnetic beads for 1 h at 4°C, as described previously. Suspensions were separated on the Baxter MaxSep apparatus. The control CAMA limiting dilution plates grew a mean of 5.9 logs, and the postpurge limiting dilution grew a mean of 1.8 logs; therefore 99.99% (4.1 logs) of tumor cells were removed. The large scale immunomagnetic separation did not affect normal hematopoietic pro-genitor cells adversely, as demonstrated by the complete recovery of CFU-GMs and BFU-Es.

**DISCUSSION**

Density gradient separation (36), lectins (17), immunotoxin (18, 20, 37), immunomagnetic beads (17, 14), and 4-hydroperoxycyclophosphamide (4-HC) (14) have been investigated with regard to their capacity to remove breast cancer cells from human bone marrow. We have used immunomagnetic beads alone or a combination of immunomagnetic beads and 4-HC acetate for patients with metastatic breast cancer and bone marrow involvement or multiple bony metastases (38). We have been able to demonstrate the presence of breast cancer cells using a two-color immunofluorescent technique in the positively selected, immunomagnetically purged bone marrow samples from the majority of our stage IV breast cancer patients treated on the purge protocol. In addition, most of the bone marrows were purged free of all detectable tumor cells (39). The combination immunopharmacological purging resulted in delayed en-graftment (40). PBPCs would hasten engraftment, but the PBPCs may be contaminated with tumor cells. The immunomagnetic purging technique can be adapted to PBPCs and removed effectively over 4 logs of contaminating breast cancer cells compared with an unpurged sample and 2.9 logs and a purged sample minus the anti-breast cancer MoAbs. The comparison with an unpurged sample is more clinically relevant.

MoAbs and complement have been used against leukemia and lymphoma (21, 41). Because it was discovered that clones of tumor cells existed that were resistant to complement-mediated lysis, immunomagnetic separation was developed (42, 43). Unlike complement-mediated purging, immunomagnetic separation does not require antibodies of a particular isotype, is not affected by anticomplementary factors associated with normal bone marrow cells, and can eliminate tumor cells expressing low levels of target antigen (44). Breast cancers show heterogeneity of antigen expression; however, multiple MoAbs can be used in immunomagnetic purging to compensate for this fact (29, 45, 46). Standard techniques exist for immunomagnetic purging of bone marrow contaminated with neuroblastoma (46) and Burkitt’s and B-cell lymphomas (47), removal of T cells from allogenic marrow to help prevent graft-versus-host disease (48), and removal of breast cancer (33) or small cell lung cancer cells (34) from the bone marrow. The efficacy of purging is difficult to prove, but there seems to be a benefit from purging for acute myeloid leukemia (49) and low-grade lymphoma (50). We demonstrated recently that the disease-free and overall survival were
worse for patients with high-risk primary breast cancer who had tumor cells in their harvested marrow compared with patients with negative marrow, and all patients were treated with high-dose chemotherapy and hematopoietic support (51). Therefore, purging may prove efficacious in the high-risk primary breast cancer setting. Purging is less likely to impact on treatment outcomes for patients with metastatic disease, because the majority of patients recur at sites of bulk disease.

The immunomagnetic purging technique could be used for each pheresis if more than one procedure were necessary. We have been able to collect enough cells with one 6-h pheresis to support a person after high-dose chemotherapy (52), which others have corroborated (53, 54). Present tumor detection techniques can detect only one tumor cell in 10^6 normal hematopoietic cells (15); therefore, it is impossible to determine whether small numbers of tumor cells contaminate the 10^10-10^11 PBPCs used to support a person. Therefore, one could argue to purge all the PBPC collections. Techniques to gene mark the purged PBPCs may provide information on the clinical efficacy of purging (55). However, a randomized trial of purging versus no purging is the only reliable way to ascertain the effect of purging.

In this study, we have shown that it is possible to remove breast cancer tumor cells from PBPCs using multiple MoAbs with immunomagnetic separation and to achieve up to 4.8 logs of tumor removal with no impairment of CFU recovery. At present, other methods such as CD34 enrichment are being explored that may help reduce the risk of reinfusing tumor cells back to the autologous transplant patient. These methods are being investigated to determine their safety and efficacy. Immunomagnetic separation can be used to eliminate a tumor from the patient’s bone marrow, and PBPCs and the purged hematopoietic cells should support a person after high-dose chemotherapy. A Phase I study for patients with metastatic breast cancer is in progress.

REFERENCES


Table 1 Log tumor cell removal and CFU recovery

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<th>Working cell concentration</th>
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Individual PBPC working cell concentration experiments using immunomagnetic purging technique showing CFU-GM and BFU-E cell recovery.
peripheral-blood progenitor cells mobilised by filgrastim (G-CSF) on granulocyte colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) on priming peripheral blood progenitor cells for use with autologous bone marrow after high-dose chemotherapy. Lancet, 1987.


Breast cancer cells are effectively purged from peripheral blood progenitor cells with an immunomagnetic technique.
