Detection and Viability of Tumor Cells in Peripheral Blood Stem Cell Collections From Breast Cancer Patients Using Immunocytochemical and Clonogenic Assay Techniques


Although peripheral blood stem cell collections (PBSC) are thought to have less tumor involvement than bone marrow (BM), the incidence of circulating tumor cells in patients with breast cancer has not been widely investigated. We prospectively investigated the incidence and viability of tumor cell involvement in PBSC and BM collections from breast cancer patients undergoing high-dose chemotherapy/hematopoietic stem cell transplantation. Paired samples of PBSC and BM from 48 patients were analyzed using an immunocytochemical technique that detects one epithelial-derived tumor cell per \(5 \times 10^5\) mononuclear cells. Immunostained tumor cells were detected in 9.6% (13/133) PBSC specimens from 9/48 (18.7%) patients and in 62.3% (38/61) BM specimens from 32/48 (66.7%) patients, a significantly higher rate than in PBSC (\(P < .0005\)).

HIGH-DOSE chemotherapy followed by autologous marrow infusion appears to be an effective treatment for some patients with locally advanced or metastatic breast cancer.\(^{14}\) However, using sensitive immunocytochemical techniques, tumor cells can be observed in histologically normal bone marrow (BM) in 20% to 45% of patients with operable disease and in 20% to 70% of patients with metastatic breast cancer.\(^{5-8}\) As a result, many patients who have multiple bone or BM metastases have not been considered eligible for autologous BM transplantation (BMT).

Recently, peripheral blood stem collections (PBSC) have been used as an alternative to BM for hematopoietic support in patients with breast cancer or hematologic malignancies who have BM disease.\(^{6-11}\) Several studies examining patients with neuroblastoma and lymphoma\(^{1,12}\) suggest that PBSC collections are less likely to contain tumor cells than BM and thus may provide a less contaminated source of hematopoietic stem cell support after high-dose chemotherapy.

The incidence and quantity of tumor cell contamination of PBSC collections in breast cancer patients has not been widely investigated.\(^{14,15}\) We prospectively examined the incidence of tumor cell contamination in paired samples of PBSC and BM collections from 48 advanced-stage breast cancer patients using a highly sensitive immunocytochemical technique. To determine whether these tumor cells were capable of clonogenic growth in vitro, tumor cell-specific clonogenic assays were performed on 58 BM or PBSC collections.

MATERIALS AND METHODS

Patient population and participating centers. Patients with histologically documented locally advanced or metastatic adenocarcinoma of the breast who were enrolled on high-dose chemotherapy programs at the participating treating institutions were eligible for this study. This protocol was approved by the Institutional Review Board for Human Investigation and each patient gave written informed consent. Extent of disease was determined by bone scans, x-rays, computed tomography (CT) scans, and routine histologic review of bilateral BM aspirates and biopsies obtained from the posterior iliac crest. Patient characteristics are listed in Table 1.

Collection of BM and PBSC specimens. Three to five milliliters of BM aspirate containing a minimum of \(1 \times 10^5\) cells/mL obtained from the posterior iliac crest were collected in sterile sodium heparin tubes and shipped at room temperature to BIS Laboratories for immunocytochemical and clonogenic assay analyses. Peripheral stem cell leukaphereses were performed at each participating institution according to protocol. In general, patients were administered chemotherapy and/or hematopoietic growth factors (either granulocyte-macrophage colony-stimulating factor [GM-CSF] or G-CSF) to enhance the mobilization of stem cells into the peripheral blood.

The geometric mean concentration of tumor cells in contaminated PBSC specimens was 0.8/10^6 mononuclear cells (range 0.33 to 2.0/10^6) compared with 22.9/10^6 mononuclear cells in BM (range 1 to 3,000/10^6, \(P < .0001\)). In culture experiments, clonogenic tumor colonies grew in 21/26 immunocytochemically positive specimens. No tumor colony growth was detected in 30/32 immunocytochemically negative specimens. Immunocytochemical detection of tumor involvement in BM and PBSC correlated significantly with in vitro clonogenic growth (\(P < .0001\)). We conclude that PBSC contain fewer tumor cells than paired BM specimens from patients with advanced breast cancer and that these tumor cells appear to be capable of clonogenic growth in vitro.

© 1993 by The American Society of Hematology.
The breast cancer cell line, CAMA-1 (generously provided by Dr. Adrian Gee, Baxter Healthcare, Santa Ana, CA), immunostained as described above was used as a positive control for each immunostaining experiment. Patient specimens were incubated with non-immune mouse serum as negative control. BM aspirates from patients without breast cancer incubated with the breast-reactive MoAb cocktail served as an additional negative control for each immunostaining experiment.

**Immunocytochemical detection and quantitation of tumor cells.** A specimen was considered immunocytochemically positive when immunostaining was observed on at least 75% of the cell membrane and cytoplasm, and morphology was consistent with a malignant cell. Immunostaining results were confirmed by two or more independent observers (A.A.R., T.J.M., D.G.K., N.E.W.). Samples were coded as “indeterminate” if immunostaining was scant, or if the morphology of positively stained cells was inconsistent with tumor cell morphology. Samples indeterminate for tumor cells were excluded in the statistical analyses.

For both PBSC and BM specimens a total of $10^6$ to $5 \times 10^6$ cells was directly examined per specimen. The total number of fields required to analyze a total of $10^6$ cells was calculated from the average number of cells present per high-power (40 x objective). If five or greater immunocytochemically positive tumor cells were detected per $10^5$ hematopoietic cells, the number of tumor cells per $10^5$ was extrapolated. If fewer than five tumor cells were detected per $10^5$ hematopoietic cells, then up to $5 \times 10^5$ cells were directly viewed and quantitated.

**Tumor cell clonogenic assay.** Mononuclear cell fractions of fresh or rapidly thawed cryopreserved PBSC and BM specimens were isolated as described above. In cases where multiple PBSC specimens were obtained, the specimens were pooled and processed as a single specimen. Mononuclear cells/mL, $5 \times 10^4$ to $10^5$, were plated in triplicate in 35-mm grid-bottom Petri dishes (Nunc, Inc, Naperville, IL) in a soft agar-based medium consisting of 30% Iscove’s modified Dulbecco’s medium (IMDM: Sigma); 20% fetal bovine serum (Sigma), 50% of a 0.6% agar solution (Sigma), 5 μg/mL human recombinant epidermal growth factor (Collaborative Research, Bedford, MA), 50 U/mL human recombinant GM-CSF (Collaborative Research), and 0.1 μg/mL human recombinant insulin-like growth factor I (Collaborative Research). Negative control plates consisted of medium without supplemental growth factors. All plates were incubated in a humidified chamber at 37°C with 7.5% CO₂ for 14 days.

| Table 1. Characteristics of Patients With Immunocytochemically Detectable BM or PBSC Contamination |
|--------------------------|------------------|------------------|
| Characteristic           | No. of Patients  | BM Positive      | PBSC Positive     |
| Total patients           | 48*              | 32               | 9                |
| Age (yrs)                |                  |                  |                  |
| 25-39                    | 14               | 11               | 2                |
| 40-49                    | 24               | 15               | 5                |
| 50-59                    | 8                | 4                | 1                |
| 60 and over             | 2                | 2                | 1                |
| Sites of metastatic disease |                |                  |                  |
| Bone                     | 30               | 22               | 6                |
| Liver                    | 4                | 3                | 2                |
| Lung                     | 12               | 9                | 4                |
| Soft tissue              | 13               | 8                | 2                |
| Bone marrow              | 18               | 15               | 5                |
| None                     | 8                | 4                | 0                |

* BM and PBSC analyses were performed on specimens obtained from 50 consecutive patients; in 2 patients immunocytochemical analyses were indeterminate, leaving 48 evaluable patients.

Processing of BM and PBSC specimens for immunocytochemical analysis. Processing of fresh BM and PBSC specimens has been reported elsewhere. Briefly, mononuclear cell fractions from BM and PBSC were isolated by Ficoll-Hypaque separation (Pharmacia, Uppsala, Sweden) and washed twice in Liebovitz L-15 medium (GIBCO/BRL, Grand Island, NY) supplemented with 15% fetal bovine serum (L-15/FBS; GIBCO/BRL). Cryopreserved specimens were rapidly thawed in a 37°C water bath, incubated with 100 μL of Deoxyribonuclease I (Sigma, St Louis, MO) at 2,000 Kunitz U/mL for 5 minutes at 37°C to prevent clotting, and washed twice with serum-free RPMI-1640 medium (GIBCO/BRL) at 1,500 rpm for 10 minutes. Mononuclear cells/mL, $1.0 \times 10^5$, were incubated at room temperature with a cocktail of monoclonal antibodies (MoAbs) in 1 mL L-15/FBS as described below.

MoAbs. A panel of IgG murine MoAbs directed against breast or glandular epithelia was used for immunocytochemical staining. Table 2 outlines the MoAbs used, the reactive cellular epitope, and commercial supplier. The MoAbs were used in a cocktail mixture. Each MoAb was used at a concentration of 5 μg/mL. Previous experiments have shown that this cocktail of MoAbs detects breast tumor cells with sensitivity and specificity levels of 99% and 98%, respectively, and is nonreactive with hematopoietic cells. In cell seeding experiments, this cocktail of MoAbs detects one tumor cell per $5 \times 10^4$ mononuclear cells (either in BM or PBSC).

Immunocytochemical staining. Immunocytochemical staining procedures have been reported previously. Briefly, after MoAb incubation in L-15/FBS, cells were washed twice with fresh L-15/FBS, spun onto slides and stored at 4°C overnight. Cytopreparations were fixed in a paraformaldehyde/methanol fixative, washed in Dulbecco’s modified PBS (GIBCO/BRL), blocked for endogenous peroxidase activity using a 50 mmol/L solution of phenylhydrazine (Sigma), blocked for nonspecific protein binding with 10% normal horse serum (Vector Laboratories, Burlingame, CA), and immunostained using the Vector avidin-biotin immunoperoxidase kit according to manufacturer’s instructions.

| Table 2. Panel of MoAbs Used for Immunocytochemical Analysis |
|-----------------------------|-----------------|------------------|
| MoAb (source)               | Reactive Antigen | Reactive Cell Type |
| MAS-385 (Accurate Chemical; Westbury, NY) | Human mammary carcinoma-associated antigen | Breast epithelium |
| SB-3 (CalTag, San Francisco, CA) | 8, 18, 19 kD cytokeratin | Glandular epithelium |
| TFS-2 (Biosdesign, Kennewick, ME) | Cellular adhesion molecule | Breast epithelium, small cell lung cancer |
| SB-6 (Accurate Chemical) | NCAM | Breast epithelium, small cell lung cancer |
| CD-11 (Accurate Chemical) | 95-150 kD glycoprotein | Monocytes, granulocytes (CFU-GM) |
Immunocytochemical detection of tumor cells. (A) Immunostaining of tumor cells in BM (arrow) shows intense immunoperoxidase reaction while hematopoietic cells are unstained. Original magnification × 400. (B) Immunostaining of single tumor cell (arrow) in PBSC collection. Original magnification × 650.

Tumor colony identification. After tumor colony quantitation, agar plates were floated onto 2-inch × 3-inch microscope slides and air dried. Tumor colonies were submitted in a blinded fashion for immunofluorescence staining to the laboratory of Dr Thomas Moss. In situ tumor colony verification was performed using immunofluorescence staining with fluorescein isothiocyanate (FITC)-labeled anticytokeratin MoAb SB-3 (Table 2). In certain cases, colony-forming unit GM (CFU-GM) colony verification was performed using immunofluorescence staining with rhodamine-labeled anti-CD11 MoAb (Table 2) that detects cells of the GM lineage. A minimum of two colonies consisting of greater than 40 cells was required to be considered positive. Tumor cell plating efficiency was calculated by dividing the number of tumor cells plated (as determined by immunocytochemical analysis) per dish by the number of tumor colonies observed per dish, with the quotient multiplied by 100.

Statistical analyses. Difference in rates of tumor cell contamination of PBSC and BM was tested using McNemar’s test of concordance. The quantity of tumor cell involvement in PBSC and BM was compared using Wilcoxon’s matched-pairs signed-rank test. Spearman’s rank correlation coefficient was used to test the relationship between the level of contamination in BM and PBSC. Chi-square and Fisher’s exact tests were used to correlate tumor cell contamination observed using immunocytochemical and tumor cell clonogenic assay techniques.

Multiple BM and PBSC collections in 11 and 28 patients, respectively, were analyzed for tumor cell involvement using immunocytochemical analysis. To perform quantitative comparisons of tumor cell concentrations in BM and PBSC collections, the number of tumor cells per 10^5 hematopoietic cells was recorded as the average of multiple collections from an individual patient.

Tumor cell clonogenic assays were performed on individual or pooled specimens. Results of each tumor cell clonogenic assay experiment, whether from individual or pooled specimens, were treated as individual samples for the statistical analyses.

RESULTS

Characteristics of patients with BM or PBSC involvement. Table 1 shows age ranges, clinical stage, and sites of metastases for the 48 evaluable patients. The majority of patients had osseous metastases, and 18 patients had BM metastases on routine histopathologic review. Examples of immunostained tumor cells in BM and PBSC are shown in Fig 1.

PBSC collections were less likely than BM to contain tumor cells in patients with either localized or metastatic breast cancer (Table 1). PBSC tumor involvement was not found in any patients with localized breast cancer. In contrast, BM aspirates from 4/8 (50%) patients with localized disease contained immunocytochemically detectable tumor cells. Patients with stage IV disease at the time of collection also were less likely to have PBSC involvement (22.5%, 9/40) than BM involvement (70%, 28/40). The differences in PBSC and BM tumor cell involvement were highly significant (P < .0001 McNemar’s test).

Among the patients with stage IV breast cancer there did not appear to be any difference in the distribution of metastases in patients between the entire cohort of patients and those with BM or PBSC contamination (Table 1). Of interest, there was a lower incidence of tumor contamination of PBSC compared with BM among all sites of metastatic disease. However, because of the small numbers of patients, this was not statistically significant.

Characteristics of the nine patients with tumor cells detectable in PBSC collections are shown in Table 3. Five of
the nine patients had tumor involvement on routine BM histology and six of the nine patients had immunocytochemically detectable tumor cells in the BM. In one patient (no. 10, Table 3) BM involvement was found on routine histologic examination but not by immunocytochemistry, indicating that the panel of MoAbs failed to detect tumor cells in this patient or that the BM aspirate sample was not representative. Subsequent immunostaining of the core biopsy specimen of the patient’s BM showed immunostained tumor cells in large clumps that were encased in a fibrotic network, suggesting that tumor cells were not aspirated into the specimen sent for immunocytochemical analysis.

Comparison of tumor involvement in BM and PBSC collections. Tumor cell involvement detected by immunocytochemistry occurred significantly less frequently in PBSC than in BM. Thirteen of 133 PBSC collections (9.8%) obtained from 9/48 patients (18.7%) had tumor cells detected by immunocytochemistry. On the other hand, tumor cells were detected in 38/61 (62.3%) BM collections obtained from 32/48 (66.7%) patients. The difference in PBSC and BM involvement was significant (P < .005, McNemar’s test). Six patients had tumor cells detected in both BM and PBSC collections. Among the 29 patients who had tumor cells detected in only one site (either BM or PBSC), significantly more patients (54.2%) were observed to have tumor cells in BM (26/48) than in PBSC collections (6.2%, 3/48; P < .005, McNemar’s test).

The numbers of specimens analyzed per patient varied from 1 to 3 for BM and 1 to 13 for PBSC. The mean number of immunocytochemically detected tumor cells in each type of collection was used for statistical analysis. In patients with multiple collections, tumor cells were not detected in all serial samples. The concentration of tumor cells detected in the immunocytochemically positive PBSC collections ranged from 1 to $4 \times 10^4$ mononuclear cells (geometric mean concentration of $0.8/10^4$ hematopoietic cells, n = 9 patients). In contrast, the concentration of tumor cells in the immunocytochemically positive BM specimens ranged from 1 to 3,000 with a geometric mean of $22.9/10^5$ cells (n = 32 patients). The difference in tumor cell concentration was statistically significant (P < .0001, Wilcoxon’s test).

PBSC involvement was not associated with histologically or immunocytochemically documented BM involvement (P = 1.0, Spearman’s rank correlation coefficient). Six of 32 (18.8%) patients with immunocytochemically detectable tumor cells in BM also had immunocytochemically positive PBSC collections. Three of 16 patients (18.8%) with immunocytochemically negative BM were found to have immunocytochemically positive PBSC collections.

Tumor cell clonogenic assay growth. Thirty-three BM specimens and 29 PBSC specimens were assayed for tumor cell clonogenic growth in vitro. Two BM samples and two pooled PBSC samples were indeterminate for detection of tumor cells by immunocytochemistry and thus were not subject to statistical analysis, leaving 58 evaluable samples. Clonogenic plating efficiency ranged from 0.4% to 14%, with a median plating efficiency of 4%. Clonogenic tumor growth was observed in both fresh and thawed, previously cryopreserved specimens.

Immunofluorescence staining verification of tumor colonies with anticytokeratin MoAb SB-3 showed intense staining of tumor colonies (Fig 2). Positive immunofluorescence staining with MoAb SB-3 was not observed in clonogenic assays that were judged by phase microscopy to be negative for colony growth in vitro. In three cases, duplicate plates were immunostained with MoAb CD-11 against monocytes and granulocytes if colony in vitro morphology was indeterminate. In none of these cases did MoAb CD-11 immunostain SB-3—positive tumor colonies, or vice versa.

As shown in Table 4, tumor cell contamination detected by immunocytochemistry correlated significantly with in vitro clonogenic growth (P < .0001, χ² test). Clonogenic tumor colony growth was observed in 21/26 immunocytochemically positive specimens, and no colony growth was observed in 30/32 immunocytochemically negative specimens (Table 4). Seventeen of 21 immunocytochemically positive BM specimens were found to contain clonogenic tumor cells, while only 2/10 immunocytochemically negative BM specimens were clonogenic (P < .05, χ² test). Similarly, 4/5 PBSC specimens that contained immunocytochemically detectable tumor cells were capable of clonogenic tumor colony growth, whereas no tumor colonies grew from the 22 immunocytochemically negative PBSC specimens (P < .0001, χ² test).

DISCUSSION

Although PBSC collections are believed to have a lower incidence of tumor involvement than BM in breast cancer patients, there are few studies to support this contention. To address this issue, we used a highly sensitive immunocytochemical technique to quantify the number of occult tumor cells in concurrently collected BM and PBSC specimens from 48 patients with stage II-IV breast cancer.

Our findings indicate that occult breast cancer cells are present less frequently in PBSC than in BM (P < .005 McNemar’s test). Furthermore, the concentration of tumor cells in immunocytochemically positive PBSC collections was significantly lower than in immunocytochemically positive BM (P < .0001, Spearman’s rank correlation coefficient). Even though patients undergoing PBSC reconstitution generally require a greater number of infused hematopoietic cells than patients receiving autologous marrow (6 to 15 × 10⁸/kg for PBSC vs 1 to 4 × 10⁹/kg for BM), this would still result in the infusion of fewer tumor cells. In addition, our data indicate that tumor cells may not be present in all PBSC pheresis collections from a single patient. Taken together, these data support the current belief that PBSC collections may be preferable to BM as a less-contaminated source of hematopoietic stem cells for autologous transplantation.

In patients with stage IV disease, the incidence of circulating tumor cells detected in PBSC collections was independent of BM tumor cell content and sites of metastatic involvement. In fact, two patients (nos. 92 and 92113, Table 3) without immunocytochemically or histopathologically detectable BM disease had PBSC tumor involvement documented by both the immunocytochemical and tumor cell clonogenic assay techniques. Thus, in some instances, BM
may provide a less-contaminated source of hematopoietic stem cells. For autologous transplantation purposes, it may be beneficial to test both BM and PBSC collections to determine which source of hematopoietic stem cells contains fewer contaminating tumor cells.

Although no study, including the study presented here, has shown that reinfused breast cancer cells can contribute to relapse, preliminary evidence in patients with non-Hodgkin’s lymphoma and leukemia suggests that the removal of tumor cells either by BM purging techniques or the use of tumor-uninvolved PBSC may be an important predictor of long-term survival. Using a more direct gene-marking technique to track the fate of reinfused leukemia cells in autologous BM harvests, Brenner et al were able to document that reinfused tumor cells were traceable to sites of disease relapse. Therefore, it may be prudent to explore the use of techniques, such as purging with chemotherapy, MoAbs, or enriching for CD34+ cells, that yield specimens least likely to contain tumor for purposes of hematopoietic reconstitution.

The tumor cell clonogenic assay technique developed for use in this study provides new evidence that tumor cells in BM and PBSC collections are viable and capable of in vitro clonogenic growth. The observed tumor cell plating efficiency (0.4% to 14%) further suggests that a significant number of tumor cells may be capable of in vivo growth. Supporting evidence that these colonies are indeed tumor-derived is provided by the anticytokeratin immunostaining of the colonies. We have also obtained positive immunostaining of tumor colonies with the breast cancer-derived MoAbs 520C9, 260F9, and 317G5 used by Franklin et al to identify BM micrometastases. Immunostaining experiments with rhodamine-conjugated MoAb CD-11, which binds to monocytes and granulocytes, showed no staining of tumor colonies.

Of concern is the possibility that tumor detection by immunocytochemical methods alone may yield false-positive and/or false-negative results. To this end, our tumor cell clonogenic assay provided additional confirmatory data of the immunocytochemical results, as tumor colony growth in vitro correlated significantly with immunocytochemical analyses in 81% of positive samples and 94% of negative samples (P < .0001, χ² test).

In two cases immunocytochemically negative specimens grew tumor colonies in the clonogenic assay. This may be due to sampling error in the immunocytochemical analysis, or lack of MoAb reactivity with the tumor cells. Subsequent immunostaining of the BM core biopsy from the first case showed clumps of tumor cells encased in a fibrotic network. This suggests that tumor cells may not have been released into the aspirated specimen. In the second case, where few hematopoietic cells (<10⁵/mL) were available for analysis by immunocytochemistry, the tumor cell clonogenic assay (where 5 x 10⁵ cells are plated per dish in triplicate) may be more sensitive in detecting low numbers of cells. This suggests that false-negative results on immunocytochemical analysis may be caused, in part, by evaluating inadequate numbers of cells. These two examples illustrate that the immunocytochemical and in vitro clonogenic assay techniques may complement each other in the detection of low numbers of tumor cells.

---

**Table 4. Comparison of Immunocytochemical and Tumor Cell Clonogenic Assays in BM and PBSC Collections**

<table>
<thead>
<tr>
<th>Cell Source</th>
<th>Immunohistochemical Assay</th>
<th>Tumor Cell Clonogenic Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM</td>
<td>Positive 21*</td>
<td>Growth 17</td>
</tr>
<tr>
<td></td>
<td>Negative 10</td>
<td>No growth 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No growth 8</td>
</tr>
<tr>
<td>PBSC</td>
<td>Positive 5*</td>
<td>Growth 4</td>
</tr>
<tr>
<td></td>
<td>Negative 22</td>
<td>No growth 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No growth 22</td>
</tr>
</tbody>
</table>

Detection of tumor cells by immunocytochemistry correlated significantly with clonogenicity (P < .0001, χ² test).

* In addition, two samples each of BM and PBSC were indeterminate for tumor by immunocytochemical analysis. In each case one of the two samples were clonogenic.
In conclusion, our data indicate that PBSC collections contain fewer tumor cells than BM, and, therefore, may be preferable as the source of hematopoietic stem cell reinfusion after high-dose chemotherapy. This finding may have additional significance given that these occult tumor cells appear to possess the potential for clonogenic growth in vitro. Further studies are required to determine whether the presence of small numbers of breast cancer cells in hematopoietic stem collections will influence the outcome of high-dose therapy programs for breast cancer patients.

ACKNOWLEDGMENT

The authors acknowledge the expert technical assistance of Chana Weintraub, Stephanie Farmer, Marina Prilutskaya, Gloria Xu, and Annie Lao.

REFERENCES

Detection and viability of tumor cells in peripheral blood stem cell collections from breast cancer patients using immunocytochemical and clonogenic assay techniques [see comments]

AA Ross, BW Cooper, HM Lazarus, W Mackay, TJ Moss, N Ciobanu, MS Tallman, MJ Kennedy, NE Davidson and D Sweet