

Detection of Carcinoma Cells in the Blood of Breast Cancer Patients

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BACKGROUND: Breast cancers shed cancer cells into the blood soon after they become invasive. We developed an assay for removing these circulating cancer cells. In this study, we wanted to determine the percentage of early stage and metastatic patients with circulating breast cancer cells.

METHODS: Twenty milliliters of blood were drawn from patients with breast cancer. Epithelial cells were removed by immunomagnetic selection and analyzed by flow cytometry, cytomorphology, and immunocytochemistry.

RESULTS: Early stage patients averaged 16 epithelial cells per 20 cc blood whereas metastatic patients averaged 122 tumor cells. Cytomorphology and immunostains confirmed that these were cancer cells. Control blood samples had 1.7 squamous epithelial cells per 20 cc blood.

CONCLUSION: This assay can identify and characterize circulating breast cancer cells. Metastatic patients had more circulating cells than early stage patients. This assay could screen high-risk patients, determine the need for and monitor response to adjuvant therapy, and detect early recurrence of breast cancer. *Am J Surg.* 2000; 180:446-449. © 2001 by Excerpta Medica, Inc.

Breast cancers have been shown to shed neoplastic cells into the circulation at the earliest stage of development.¹⁻⁵ The natural history of these cells and their ability to establish metastases have not been clearly shown.^{6,7} Recently, breast cancer cells have been detected in the bone marrow of selected patients and this was found to have significant prognostic importance.⁸ Since early diagnosis is the key to successful treatment of breast cancer and shedding of tumor cells is an early event in breast cancer, it may be possible to detect cancer cells in the blood before the primary tumor is large enough to be detected by physical examination or mammography.

Several investigators have used polymerase chain reaction (PCR) to detect tumor cells^{9,10}; however, the sensitivity and ideal technique for PCR have not been stan-

dardized. We have developed a highly sensitive assay for detecting and characterizing circulating carcinoma cells.⁵ In this study we wanted to determine the percentage of patients with circulating breast cancer cells in both early stage and metastatic disease, and to characterize these cells by cytomorphology and immunocytochemistry.

METHODS

Collection of Blood Specimens

After appropriate informed consent was obtained, 10 to 20 mL blood were drawn from patients with a primary diagnosis of breast cancer into Vacutainer EDTA tubes (Becton Dickinson, San Jose, California). Samples were also drawn from hospital personnel who volunteered to serve as controls. The samples were processed within 24 hours of collection. Patient age, sex, date of diagnosis, therapeutic interventions, clinical status, and biopsy report were retrieved from the patients' charts. The protocol was approved by the institutional review boards of the collaborating institutions.

Sample Preparation for Flow Cytometric Analysis

We used an immunomagnetic sample preparation procedure developed by Immunicon Corporation that can identify 1 epithelial cell in 20 mL peripheral blood. Monoclonal antibodies (mAbs) against the epithelial cell adhesion molecule (EPCAM) are broadly reactive with tissues of epithelial cell origin. The epithelial cell antibody (kindly provided by D. Herlyn Wistar Institute, Philadelphia, Pennsylvania) was coupled to an iron-containing compound, "ferrofluid," to allow magnetic separation. Blood was incubated with the EPCAM/ferrofluid for 15 minutes and was then placed in a magnetic field. The magnetically separated cells were resuspended in a solution containing fluorescence-conjugated anticytokeratin (epithelial cell marker) and anti-CD45 (lymphocyte control marker) for 15 minutes. The cells were analyzed on a FACScan flow cytometer (Becton Dickinson). Data analysis included size, granularity, red fluorescence (epithelial marker), and green fluorescence (lymphocyte marker). Reagents for flow cytometry were kindly provided by Becton Dickinson.

For immunocytochemistry analysis, cytospin preparations were made from immunomagnetic separated samples. Immunostains for epithelial cells (cytokeratin) and breast cancer cells (MUC-1 glycoprotein) were used to stain the slides.

For cytomorphology studies, a cytospin prepared slide from each of the specimens was stained with hematoxylin and eosin and examined for the cytomorphologic features of cancer cells.

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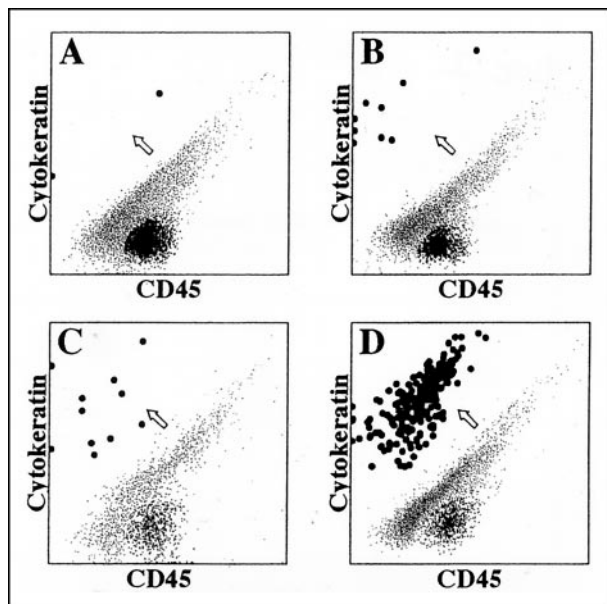


Figure 1. Flow cytometric analysis using cytokeratin (epithelial cell marker) on the y-axis and CD45 (lymphocyte marker) on the x-axis. Panel A is blood from a control patient with 2 epithelial cells (large black dots), panels B and C are from early breast cancer patients with a few epithelial cells, and panel D is from a metastatic breast cancer patient with many epithelial cells.

TABLE I

Number of Patients/Controls with Circulating Epithelial Cells and Quantity of Those Cells

	Number with Circulating Epithelial Cells/N	Average Number of Cells/20 cc Blood (Range)
Controls	12/23 (52%)	1.7 (0-5)
Stage I/II patients	22/23 (96%)	16 (5-31)*
Stage IV patients	11/11 (100%)	122 (55-600)*

P < 0.01 by *t* test.

RESULTS

We analyzed the blood of 34 patients with breast cancer. There were 23 stage I/II and 11 stage IV patients, and 23 control patients. We first examined immunomagnetically separated nucleated cells from the blood of patients with breast cancer and from controls for the presence of epithelial cells (cytokeratin positive/CD 45 negative) using flow cytometry (see **Figure 1**). Ninety-five percent of early stage breast cancer patients had epithelial cells in their blood with an average of 16 cells per 20 cc of blood (range 5 to 31). All patients with metastatic breast cancer had circulating epithelial cells with an average of 122 epithelial cells per 20 cc of blood (range 55 to 600). The control patients' blood samples averaged 1.7 epithelial cells per 20 cc, ranging from 0 to 5 (see **Table I**).

Next we wanted to determine if the epithelial cells identified by flow cytometry could be shown to be cancer cells. Blood from control and breast cancer patients underwent immunomagnetic selection and these cells were then spun onto slides. The cytopsin slides were examined by standard histologic criteria, as well as immunostains with an-

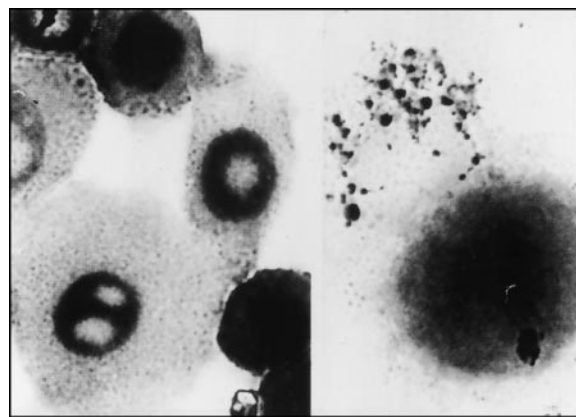


Figure 2. Epithelial cells (left) and circulating breast cancer cells (right).

ticytokeratin (epithelial cell marker) and anti-MUC-1 (breast cancer marker) mAbs. The cytopsin slides were examined in a blinded fashion. Immunostains showed them to be epithelial cells (cytokeratin positive) and tumor cells (MUC-1 positive). The cytomorphology was consistent with breast cancer cells similar to their primaries. The control patients had epithelial cells that appeared to be squamous epithelium and were negative for MUC-1 (see **Figure 2**).

COMMENTS

Ever since a blood test for prostate cancer was developed (prostate-specific antigen, or PSA) the detection of early prostate cancer has increased dramatically. This has created additional controversy due to the wide variability in clinical behavior and in the treatment of prostate cancer in general. Conversely, breast cancer tends to occur in a younger population, is generally more aggressive, and there is less debate as to the best treatment for breast cancer. Screening tests for breast cancer are crude at best, with physical examination rarely finding tumors until they are at least 1 cm, with mammography somewhat better. There is a general consensus that the earlier a breast cancer is detected and treated, the better the long-term survival. Thus, the need for an early breast cancer test is obvious. Our immunomagnetic separation technique is extremely sensitive at detecting small numbers of circulating epithelial cells. Combining this technique with immunochemistry and cytologic examination, we are then able to differentiate between normal epithelial cells and breast cancer cells (perhaps the epithelial cells found in controls were from the needle penetrating the skin to draw the blood). The sensitivity of this blood test may make it useful as a screening test for high-risk patient populations.

This blood test could also be useful for patients undergoing adjuvant chemotherapy, both to determine if there is a need for adjuvant therapy as well as to monitor the effectiveness of chemotherapy. Recently, another study demonstrated circulating cancer cells in 95% of early stage I/II breast cancer.¹¹ Follow-up at 48 hours indicated that 70% of these patients had cleared their circulating cells. The remaining 30% of patients with circulating cells at 48 hours still had circulating cells at 2 weeks despite removal of all known cancer. It can be hypothesized that these

patients had residual disease that was still shedding cells into the blood. These micrometastases are clinically undetectable; however, the sensitivity of the immunomagnetic assay is such that these shed cancer cells can be detected and observed over time. These may be the patients who benefit from adjuvant chemotherapy. We know that some but not all patients with early stage breast cancer benefit from chemotherapy but we currently have no way to determine who will benefit. This assay may allow us to determine which patients have micrometastatic disease (the treatment of micrometastases is the basis for adjuvant chemotherapy) and which patients do not. In addition, adjuvant chemotherapy is often administered over a predetermined number of cycles with specific treatment regimens. This test may allow us to determine the effectiveness of the adjuvant chemotherapy and to alter the regimen if breast cancer cells do not clear from the circulation. This is analogous to switching regimens in patients with metastatic disease who do not have shrinkage of tumor based on imaging or laboratory evaluations.

Lastly, there are currently millions of breast cancer survivors in America. We routinely do multiple tests (complete blood count, chest radiograph, liver function test, bone scan) to try to detect recurrences at the earliest possible time. These tests generally pick up metastases only after they are large and well established. Perhaps this assay could be used to follow up patients after therapy to detect recurrences when they are early and subclinical. This is when most oncologists believe treatment of metastatic disease is most effective.

Much more research needs to be done in this area. However, the prospect of a breast cancer blood test is encouraging to clinicians and patients.

DISCUSSION

Dr. Kent C. Westbrook (Little Rock, AR): During the last 50 years, breast cancer has come to be thought of as a systemic disease, based as was indicated on work by Fisher and the NSABP people. By systemic disease, it has been meant that cancer cells are shed into the lymphatics and the blood stream when the cancer is still small. Frequently, even with early disease, node-negative disease, and small lesions, these cells have implanted and started growth at distant sites before diagnosis is made. Hence, these patients are said to have systemic disease.

On the other hand, as the authors mentioned, most patients that are cured of breast cancer today, are cured by surgery and radiation therapy. They're cured by local treatments, not by systemic treatments. If you take 100 patients with invasive breast cancer, about 50 of them will be cured by their local treatments. About 15 will be cured by adjuvant therapy, and about 35 will not be cured of their disease. These observations point out that there are two major questions in trying to really decide how to treat patients right now.

Number 1: How can we avoid chemotherapy in those patients who don't need it?

REFERENCES

1. Brandt B, Roetger A, Heidl S, et al. Isolation of blood-borne epithelium-derived c-erbB-2 oncoprotein-positive clustered cells from the peripheral blood of breast cancer patients. *Int J Cancer*. 1998;76:824-888.
2. Eaton MC, Hardingham JE, Kotasek D, Dobrovic A. Immunobead RT-PCR: a sensitive method for detection of circulating tumor cells. *Biotechniques*. 1997;22:100-105.
3. Hildebrandt M, Mapara MY, Korner JJ, et al. Reverse transcriptase-polymerase chain reaction (RT-PCR)-controlled immunomagnetic purging of breast cancer cells using the magnetic cell separation (MACS) system: a sensitive method for monitoring purging efficiency. *Exp Hematol*. 1997;25:57-65.
4. Naume B, Borgen E, Beiske K, et al. Immunomagnetic techniques for the enrichment and detection of isolated breast carcinoma cells in bone marrow and peripheral blood. *J Hematother*. 1997;6:103-114.
5. Racila E, Euhus D, Weiss AJ, et al. Detection and characterization of carcinoma cells in the blood. *Proc Natl Acad Sci USA*. 1998;95:4589-4594.
6. Filder I. Metastasis: quantitative analysis of distribution and fate of tumor emboli labeled with 125 I-5-iodo-27 deoxyuridine. *J Natl Cancer Inst*. 1970;45:773.
7. Butler TP, Giuliano PM. Quantitation of cell shedding into efferent blood of mammary adenocarcinoma. *Cancer Res*. 1975;35:512-516.
8. Braun S, Pantel K, et al. Cytokeratin-positive cells in the bone marrow and survival of patients with stage I, II, or III breast cancer. *NEJM*. 2000;342:525-533.
9. Pantel K, Schlimok G, Braun S, et al. Differential expression of proliferation-associated molecules in individual micrometastatic carcinoma cells. *J Natl Cancer Inst*. 1993;17:1419-1424.
10. Gerhard M, Juhl H, Kalthoff H, et al. Specific detection of carcinoembryonic antigen-expressing tumor cells in bone marrow aspirates by polymerase chain reaction. *J Clin Oncol*. 1994;4:725-729.
11. Krag DN, Ashikaga T, Moss TJ, et al. Breast cancer cells in the blood: a pilot study. *Breast J*. 1999;5:354-358.

And number 2: How can we identify patients who are going to fail chemotherapy?

The authors have presented a technique that may help answer those questions. They collected peripheral blood, they isolated epithelial cells, they counted the cells, and they identified them as malignant cells. I think that's very clear in their paper. They showed that even early stage breast cancer patients had circulating malignant cells. So this is a very elegant way to demonstrate that breast cancer is a systemic disease.

I would like to pose three questions to the authors to try to determine whether they really think there's going to be any clinical application of this.

Number one, how complex and expensive is the test? Is it going to be simple enough that it can be used in the average hospital?

Number two, is this really superior to a simple bone marrow aspiration? And is it more reliable than a bone marrow aspiration?

And number three, realistically, what do you think the possibility is that this can answer those first two questions: who's going to be cured without chemotherapy and doesn't need it, and who's going to fail adjuvant chemotherapy, and needs to have it changed?

CLOSING

Dr. Peter D. Beitsch: First of all, is it complex and expensive? Well, yes. Is it going to be available in every hospital? No. Can you order this test today? Yes, you can call Impath Bis. and send them a tube of blood and get the answer back. Today it's available commercially, but it is complex, and I'm sure it's not cheap.

Number two, is it superior to bone marrow aspiration? Well, I think bone marrow aspiration is just another way to draw blood, and I think if you ask most people how they would like some blood drawn, I think most people would take a vein over the bone marrow. But, I think more work on bone marrow "micromets" really needs to get done because it may just be a reflection of circulating cancer cells.

And, is this test going to allow us to figure out who is

cured and who is not? It's not actually addressed in this paper, but I can tell you we published an article in the December issue of the Breast Journal, where we looked at perioperative blood draws in early stage breast cancer patients. And we found that, just like this paper, 95 percent of early stage breast cancer patients had circulating cells. We did 8 postoperative blood draws, and by 48 hours that number was down to 30 percent. And then we drew blood at 7 days and at 14 days, and at 14 days 30 percent of the same patients still had circulating cancer cells. I think the provocative part about that paper is, those may be the patients that need chemotherapy.

There's a lot more to learn, but I think you're going to hear a lot more about circulating tumor cells, and I think it's going to be a good marker for us.