Detection and Clinical Implications of Early Systemic Tumor Cell Dissemination in Breast Cancer

Klaus Pantel, Volkmar Müller, Margherita Auer, Nathalie Nusser, Nadia Harbeck, and Stephan Braun

Abstract

Blood-borne distant metastasis is the leading cause of cancer-related death in breast cancer. The onset of this fundamental process can now be assessed in cancer patients using ultrasensitive immunocytochemical and molecular assays able to detect even single metastatic cells. Analyses of bone marrow (BM) samples show that disseminated cells are present in 20–40% of primary breast cancer patients without any clinical or histopathological signs of metastasis. The common homing of circulating breast cancer cells in BM is indicative for systemic tumor cell spread and predictive for growth of overt metastases in relevant organ sites such as bone, lung, or liver. Recent clinical studies involving more than 3000 breast cancer patients demonstrated that the presence of tumor cells in BM at primary diagnosis is an independent prognostic factor for unfavorable clinical outcome. To date, sampling of BM, however, is not a routine procedure in clinical management of breast cancer patients. Therefore, several research groups have developed sensitive assays for detection of circulating tumor cells in peripheral blood. Studies evaluating the clinical relevance of these blood assays are ongoing. Here, we will review the existing tumor cell assays and discuss their current clinical relevance and perspectives for the clinical management of breast cancer patients.

Introduction

The majority of cancers in industrialized countries are solid tumors derived from epithelial tissues, such as carcinomas of the breast, lung, gastrointestinal tract, and prostate. Although the presence of lymph node (LN) metastasis is a negative prognostic factor for breast cancer and other cancers, it is still not possible to reliably identify those patients who will eventually relapse with metastatic disease only by their LN status at primary therapy, indicating that other ways of metastatic tumor cell spread also play an important role. Advances in the development of immunocytochemical and molecular assays now enable specific detection of metastatic tumor cells even at the single cell stage and thus allow us to address the important question of systemic tumor cell dissemination as one of the first crucial steps in the metastatic cascade. Using these technologies, it has become evident that 20–40% of patients with various epithelial tumors (e.g., carcinomas of the breast, prostate, colon, or lung) harbor occult metastatic cells in their bone marrow (BM) even in the absence of any LN metastases (stage N0) and clinical signs of overt distant metastases (stage M0). However, these techniques for detection of metastatic cells are not yet established in clinical routine practice, and no international consensus has been reached to recommend a single standardized protocol as benchmark technology.

In breast cancer, recent guidelines for adjuvant systemic therapy in case of a negative LN status result in treatment recommendations for over 90% of patients (1). The risk of tumor relapse in these patients is considered high enough to recommend adjuvant therapy, even though up to 70% of early-stage breast cancer patients are cured by locoregional surgery alone. Therefore, the availability of additional factors enabling individual risk assessment is desirable to improve identification of patients at risk for relapse. Several studies in breast cancer suggested that the presence of disseminated tumor cells in BM represents an additional clinical marker that may be capable of identifying those patients who are cured by surgery alone. One of the intriguing opportunities of this marker might therefore be its use for clinical decision making in risk-adapted adjuvant treatment strategies. Another important and (in comparison with other markers) unique application might be the monitoring of therapeutic efficacy in the adjuvant setting with no measurable disease.

In this overview, we discuss usefulness and clinical relevance of immunological and molecular analyses in diagnosis and characterization of disseminated tumor cells (DTCs) in blood and BM of breast cancer patients.

Methods for Tumor Cell Detection

Immunocytochemistry. Using conventional histopathological techniques at time of primary diagnosis without clinical signs of metastatic bone disease, the likelihood of identifying isolated breast cancer cells in BM is as low as 4% (2). These findings imply that histological evaluation is not sensitive enough for the indicated purpose.

To date, most experience with BM screening for occult metastatic breast cancer cells exists for immunocytochemical
analyses using Ficoll density gradient centrifugation for tumor cell enrichment. As shown in Table 1, numerous studies reported a strong prognostic impact of the presence of DTCs (3–11). Other reports (12–17) failed to do so; most of them are, however, more than 10 years old. One reason for discrepant results is a substantial methodological variation (e.g., sensitivity and specificity of detection antibody, lower detection rate of BM biopsy as compared with BM aspiration, and considerable variation in the number of cells analyzed), resulting in a wide range of detection rates within the study populations (Table 1). Nevertheless, the five most recent studies (3–7) comprising a total of 2506 patients consistently reported that the presence of DTCs in BM has a strong prognostic impact on patient survival (Table 1).

However, even in these studies, at least three confounding technical factors varied considerably: (a) consistent and blinded analysis of noncarcinoma control patients; (b) diversity of antibodies used for identification of epithelial cells in BM; and (c) number of cells analyzed per patient sample.

Sufficient methodological validation of the detection antibodies has only been reported for anti-cytokeratin (CK) antibodies (18–20). The rare occurrence of single CK-positive cells in aspirates of noncarcinoma control patients (3) points to minimal, yet acceptable, technical variations within a biological system. The resulting risk of false positive findings in cancer patients can be minimized by using morphological criteria in addition to immunostaining for diagnosis of DTCs. Additional justification for using CK-specific antibodies in screening assays for occult breast carcinoma cells can be derived from several recent studies, describing phenotypic characteristics of CK-positive cells as similar to those usually found in malignant solid tumors (Refs. 21–24; Fig. 1).

The immunocytochemical technique for detection of rare events is very dependent on the reaction parameters. In this context, the number of cells analyzed and the number of non-cancer control patients included is important information that is not provided in some reports on the prognostic relevance of DTCs (Table 1). Therefore, both the International Society of Cell Therapy and the National Cancer Institute have recognized the need for standardization of the immunocytochemical assay and for its evaluation in prospective studies (25, 26). The criteria for a good tumor cell detection method are listed in Fig. 2. On the basis of data available from published methodological analyses (3, 18, 19, 25), such a standardized assay may consist of a specificity-proven, anti-CK monoclonal antibody (i.e., A45-B/3) and a sufficient sample size (i.e., 2 × 10^6 mononuclear cells/patient) obtained from two aspiration sites. The use of new automated devices for the microscopic screening of immunostained slides may help to read slides more rapidly and to increase reproducibility of the read-out process by reducing subjectivity and the need for large experience with evaluating cells (27–32).

Some of the discordant results from studies examining the role of DTCs might also be due to the low frequency of these cells. New enrichment techniques based on improved density gradient methods, such as the OnkoQuick system described recently (33, 34), and immunomagnetic procedures (35, 36) may increase the sensitivity of immunocytochemical approaches and might therefore be helpful to obtain more consistent data on the clinical significance of disseminated tumor cells. Immunomagnetic enrichment using antibody-based systems can be achieved by positive or negative selection. Tumor cells can be selected by beads coated with antibodies against tumor-associated antigens (positive selection), or normal blood cells in the preparation can be depleted by using beads coated with antibodies against hematopoietic cell antigens [negative selection (36–41)]. All these enrichment strategies have the additional advantage that the tumor cells are still viable and can be used for additional studies including the propagation of malignant cells in vitro (42). However, clinical studies will be required to determine whether the novel enrichment techniques are superior to “standard” methods, such as Ficoll gradients.

A considerable advantage of the immunocytochemical approach is the possibility to characterize DTCs by multiple staining or the combination of immunostaining and fluorescence in situ hybridization analysis (Fig. 1). Although our double labeling results show a considerable phenotypic heterogeneity.

Table 1 Reports demonstrating a prognostic relevance of disseminated tumor cells in bone marrow of breast cancer patients without overt metastases (TNM stage M0)

<table>
<thead>
<tr>
<th>Study (ref. no.)</th>
<th>Antigens</th>
<th>Preparation</th>
<th>Technique</th>
<th>No. of cells analyzed</th>
<th>No. of patients</th>
<th>Detection rate</th>
<th>No. of positive noncancer controls</th>
<th>Prognostic value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Landys et al. (9)</td>
<td>CK^a</td>
<td>Biopsy</td>
<td>IHC</td>
<td>n.s.</td>
<td>128</td>
<td>19%</td>
<td>n.s.</td>
<td>DFS^b, OS^b</td>
</tr>
<tr>
<td>Harbeck et al. (10)</td>
<td>CK, TAG-12</td>
<td>Cell smears</td>
<td>ICC</td>
<td>n.s.</td>
<td>100</td>
<td>38%</td>
<td>n.s.</td>
<td>DFS^b, OS^b</td>
</tr>
<tr>
<td>Diel et al. (4)</td>
<td>TAG-12</td>
<td>Cell smears</td>
<td>ICC</td>
<td>4–5 × 10^6</td>
<td>727</td>
<td>43%</td>
<td>0/21</td>
<td>DFS^b, OS^b</td>
</tr>
<tr>
<td>Mansi et al. (7)</td>
<td>EMA</td>
<td>Cell smears</td>
<td>ICC</td>
<td>n.s.</td>
<td>350</td>
<td>25%</td>
<td>n.s.</td>
<td>DFS, OS</td>
</tr>
<tr>
<td>Gebauer et al. (5)</td>
<td>EMA, CK</td>
<td>Cell smears</td>
<td>ICC</td>
<td>n.s.</td>
<td>393</td>
<td>42%</td>
<td>0/20</td>
<td>DFS^b, OS</td>
</tr>
<tr>
<td>Cote et al. (11)</td>
<td>CK</td>
<td>Cell smears</td>
<td>ICC</td>
<td>n.s.</td>
<td>49</td>
<td>37%</td>
<td>n.s.</td>
<td>DFS</td>
</tr>
<tr>
<td>Braun et al. (3)</td>
<td>CK</td>
<td>Cytospins</td>
<td>ICC</td>
<td>2 × 10^6</td>
<td>552</td>
<td>36%</td>
<td>2/191</td>
<td>DFS^b, OS^b</td>
</tr>
<tr>
<td>Gerber et al. (6)</td>
<td>CK</td>
<td>Cytospins</td>
<td>ICC</td>
<td>n.s.</td>
<td>484</td>
<td>31%</td>
<td>n.s.</td>
<td>DFS^b, OS^b</td>
</tr>
<tr>
<td>Braun et al. (8)</td>
<td>CK</td>
<td>Cytospins</td>
<td>ICC</td>
<td>2 × 10^6</td>
<td>150</td>
<td>29%</td>
<td>n.s.</td>
<td>DFS^b, OS^b</td>
</tr>
<tr>
<td>Vannucchi et al. (52)</td>
<td>CK19</td>
<td>Cell suspension</td>
<td>RT-PCR</td>
<td>1–1.5 × 10^6</td>
<td>33</td>
<td>48%</td>
<td>6/43</td>
<td>DFS</td>
</tr>
</tbody>
</table>

^a CK, cytokeratin; DFS, disease-free survival; DDFS, distant disease-free survival; EMA, epithelial membrane antigen; ICC, immunocytochemistry; IHC, immunohistochemistry; OS, overall survival; n.s., not stated; RT-PCR, reverse transcription-PCR; TAG-12, tumor-associated glycoprotein-12.

^b Prognostic value supported by multivariate analysis.
of DTCs in BM, particular antigens, such as HER2/neu, epithelial cell adhesion molecule, urokinase-type plasminogen activator receptor, and extracellular matrix metalloproteinase inducer EMMPRIN, are frequently expressed in DTCs (Fig. 3). These antigens are interesting potential targets for systemic adjuvant therapies aimed at eliminating minimal residual disease in breast cancer patients.

**Polymerase Chain Reaction (PCR).** The lack of a unique DNA marker for breast cancer has induced the search for tumor-associated mRNA transcripts that can be amplified a millionfold by a reverse transcription-PCR (RT-PCR) reaction. The major limiting factor for detection of single tumor cells by RT-PCR is illegitimate low-level transcription of tumor-associated or epithelial-specific genes in normal cells (43–47). Moreover, deficient expression of the marker gene in DTCs may lower the actual sensitivity of the RT-PCR assay in vivo as compared with experimental model systems that use tumor cell lines for sensitivity estimation (45). The studies conducted thus far applied CK, mucin, carcinoembryonic antigen, /H9252-subunit of human chorionic gonadotropin, mammaglobin, epidermal growth factor receptor, and several other mRNA markers (48–55).

Quantitative real-time RT-PCR provides interesting prospects for better cutoff between mRNA expression in normal cells and tumor cells, thereby increasing the specificity of the RT-PCR approach. In addition, exact quantification of the tumor cell load in individual samples might also be an important goal (53, 56–59). However, the amount of marker transcript per tumor cell may vary considerably between tumor cells of an individual patient and also among tumor cells of different patients. Thus, it might be difficult to interpret the PCR results and distinguish between changes in tumor cell numbers or changes in expression levels. The situation becomes even more complex if the RT-PCR test is used to monitor therapy-induced changes in minimal residual disease because the therapy might affect the expression level of the marker transcript in tumor cells. One possibility to circumvent this serious problem is the use of a multimarker RT-PCR test with independent RNA markers belonging to different gene families (56, 60).

In addition to the analysis of nucleic acids isolated from cellular fractions (e.g., mononuclear blood cells), tumor-specific circulating nucleic acids can be detected in serum and plasma of cancer patients using microsatellite alterations of DNA or te-
lomerase mRNA as markers (61–64). The presence of such nucleic acids even several months after primary surgery for breast cancer (65) suggests that minimal residual disease might also be detectable by such elaborate methods.

Clinical Relevance of Tumor Cell Detection
Detection of Tumor Cells in BM. Thus far, studies on the prognostic value of DTCs in BM are based mainly on immunocytochemical data (Table 1). An important initial question was whether the presence of epithelial antigen-positive cells is correlated with established risk factors in breast cancer, such as tumor size or LN involvement. Diel et al. (4) found a significant correlation between BM positivity, as assessed by anti-tumor-associated glycoprotein-12 immunocytochemistry, and tumor size, nodal status, histopathological tumor grading, as well as postmenopausal status. The London Ludwig Cancer Institute Group described that the presence of epithelial membrane antigen (EMA)-positive cells in BM was significantly related to LN involvement, peritumoral vascular invasion, and primary tumor size (66). Studies using different anti-CK monoclonal antibodies demonstrated merely a tendency toward correlation between detection of CK-positive cells in BM and locoregional LN involvement (20, 67). Applying the broad-spectrum anti-CK monoclonal antibody A45-B/B3 for DTC detection (3), a significant association of such cells in BM with the diagnosis of inflammatory breast cancer, tumor size, extensive LN metastasis of ≥10 nodes, and tumor grade was reported.

To assess the clinical significance of DTCs in BM, follow-up studies were initiated. Using a polyclonal EMA antibody, Mansi et al. (7) detected metastatic cells in 25% of BM samples. In their 6-year follow-up analysis, univariate statistics revealed that the immunocytochemical finding predicts for an increased rate of relapse in bone and other distant sites, as well as decreased overall survival. Multivariate analysis after both 6 years of clinical follow-up (68) and >12 years of clinical follow-up (7) indicated that the prognostic impact of EMA-positive cells was not independent of established risk factors, such as tumor size, grade, and LN status. Using another mucin marker, tumor-associated glycoprotein-12, Diel et al. (4) reported a detection rate of 43% in a cohort of 727 primary breast cancer patients. After a median follow-up time of 36 months (range, 3–108 months), the presence of tumor-associated glycoprotein-12-positive cells was described as being superior to axillary LN status, tumor stage, and tumor grade as an independent prognostic indicator for both metastasis-free and overall survival.

Using different mixtures of monoclonal antibodies to cell surface antigens and CKs, Cote et al. (11), Harbeck et al. (10), and Gebauer et al. (5) were able to derive prognostic information from the presence of occult metastatic cells in BM. A potential disadvantage of some of the earlier studies is substantial methodological variation. Therefore, a prospective two-center study in 552 primary breast cancer patients was initiated, using a more validated immunoassay (18, 19). The test results could be reproduced independently at both study centers (3); multivariate regression analysis verified that the presence of DTCs in BM predicts poor prognosis independent of LN status (3). These findings demonstrate for the first time that by using an identical assay for detection of DTCs, reproducible results can be generated.

In this context, it is interesting to mention that the presence of LN (micro)metastases does not necessarily correlate with the presence of occult metastatic cells in BM (6, 8). This lack of correlation may be explained by the presence of two independent metastatic pathways in breast cancer, lymphogenic and hematogenic spread, determined by different sets of genes (69).

Circulating Tumor Cells in the Peripheral Blood (PB). PB would be an ideal source for the detection of disseminated tumor cells because of an easy sampling procedure. The presence of malignant cells in PB was already described several decades ago (70, 71). A number of more recent reports have
confirmed the malignant nature of the detected cells using cytogenetic or molecular analyses (72–74). From model systems, it was estimated that about 10⁷ tumor cells/g tumor tissue are shed daily into the blood, although such model calculations might overestimate the number actually shed in vivo (75–77). Recent studies also showed that intraoperative shedding of tumor cells into the circulation may take place (78–82). However, for tumor cells, blood is only a temporary compartment, and it is not known whether a significant part of circulating tumor cells survives and is subsequently capable of forming detectable metastases. One report showed that a large number of circulating tumor cells in patients with breast cancer are apoptotic and that these cells might therefore be unable to settle in secondary organs (83). However, it was also shown that free DNA from apoptotic bodies can be transferred to other cells, suggesting that such DNA fragments might not be completely harmless (84, 85).

The prognostic significance of circulating tumor cells is much less clear than for DTCs in BM. In breast cancer, it was shown that the presence of tumor cells in blood as detected by immunocytochemical or molecular methods correlated with stage and course of the disease (86, 87). In addition, there is also one more recent report showing a prognostic impact of circulating tumor cells detected in the blood of breast cancer patients by CK19 RT-PCR (88). For other solid tumors, several reports have also shown a prognostic impact of circulating tumor cells in blood (89–93). However, it remains debatable whether these studies are empowered enough to answer the question of prognostic impact with a reasonably low β-type error, in view of the relatively small study populations and sole focus on Ps for α-type error.

Genetic Characterization of Disseminated Cancer Cells

Recent technical developments have made it possible to examine the genome of single disseminated cancer cells. Using a combination of immunocytochemistry and fluorescence in situ hybridization, several groups have reported numerical chromosomal aberrations in CK-positive cancer cells in BM, indicating the malignant origin of these disseminated cells (73, 94, 95). The availability of new protocols for the amplification of the whole genome (96–98) has enabled a more detailed analysis of disseminated tumor cells. Using single cell comparative genomic hybridization, previous studies (74, 97, 99) were able to demonstrate that CK-positive cells in BM of breast cancer patients without clinical signs of overt metastases are genetically heterogeneous. Surprisingly, these cells showed little resemblance to their respective primary tumor (99). Besides potential technical problems, a possible interpretation of this finding is that the disseminated cancer cells may have separated from their primary tumor at an early stage and evolved independently, driven by the specific selective pressures of the BM environment. Although this hypothesis adds a new perspective to the currently debated models of metastasis (100), the study by Schmidt-Kittler et al. (99) did not address the viability and proliferative potential of the disseminated cells. Therefore, it is unclear whether the cells analyzed are really the cells that evolve into metastases and whether the observed genomic alterations are of any clinical relevance (101).

A different strategy includes the assessment of the viability and proliferative potential of disseminated cancer cells in conjunction with molecular single cell analysis (95). The proliferative capacity of CK-positive tumor cells in BM of cancer patients can be assessed using special culture conditions, and the proliferative potential of these cells determines the clinical outcome (95). Hence, molecular analysis of these cultured cells may be superior for the detection of genetic changes relevant for the development from latent disseminated cells into overt metastasis.

Even after short-term culture, the number of tumor cells available for molecular analysis is still small (on average, between 1,000 and 10,000 cells), which limits further functional analyses of these cells. In this context, permanent cell lines developed from disseminated cells in BM might be helpful tools. These cell lines show typical expression and genomic characteristics of epithelial tumor cells in situ (21, 102) and may therefore serve as models for disseminated cancer cells.

Conclusions and Perspectives

Various immunocytochemical and molecular methods have been applied to detect occult hematogeneous tumor cell spread in breast cancer patients. International consensus is now urgently needed regarding quality control issues and criteria for acceptable technical assay performance, such as false negative and false positive rates, for clinically applicable assays to permit comparisons between different assay platforms. Finally, marker implementation into current risk classification systems, such as the Tumor-Node-Metastasis (TNM) Classification System, is needed. A useful proposal has recently been made by the International Union Against Cancer (103, 104). The most recent TNM classification for breast cancer (105) does not qualify the presence of single cancer cells in PB or BM as metastasis (stage M₀), but it optionally reports the presence of such cells together with their detection method, e.g., M₀(i+ ) for immunocytochemical detection or M₀(mol+ ) for detection by molecular methods.

Beyond merely adding another prognostic factor to the plethora of such markers in breast cancer, the potential of occult hematogeneous tumor cell spread as a tool for prediction or monitoring the efficacy of systemic therapy needs to be emphasized. In contrast to LNs, which are generally removed at primary surgery, BM and blood can be obtained repeatedly in the postoperative course of the patient. For example, with regard to adjuvant systemic therapy, therapeutic efficacy can be assessed thus far only retrospectively in large-scale clinical trials after an observation period of at least 5 years. Consequently, progress in this form of therapy is extremely slow and cumbersome, and, in addition, therapy is difficult to tailor to tumor response in an individual patient. The potential of a surrogate marker assay that permits immediate assessment of therapy-induced cytotoxic effects on occult metastatic cells is therefore evident. Prospective clinical studies are now required to evaluate whether eradication of DTCs in BM and blood after systemic therapy translates into a longer disease-free period and overall survival.

Our previous study showed that CK-positive DTCs in BM
rarely proliferate (i.e., they are negative for the proliferation marker Ki-67) at the time of primary diagnosis (22). Cytotoxic chemotherapy regimens might therefore fail to eliminate dormant, nonproliferating tumor cells, which may explain metastatic relapse even after high-dose chemotherapy (41, 106, 107). In addition, chemotherapy reduces immunocompetent cells in BM (108). Thus, complementary biological therapies are urgently needed for the elimination of DTCs. In two preliminary studies using antibody-based immunotherapy, reduction of DTCs in BM was observed (109, 110). However, repeated BM sampling might not be easy to implement into clinical study protocols for breast cancer. Serial examinations of blood for circulating tumor cells or tumor cell-associated nucleic acids might be more acceptable for most patients and clinical investigators than repeated BM aspirations. The detection and characterization of circulating tumor cells in PB of cancer patients might be more acceptable for most patients and clinical investigations than repeated BM aspirations. The detection and characterization of circulating tumor cells as a surrogate marker for clinical therapeutic targets such as the HER-2/neu oncogene product can also lead to strategies for evaluation of therapeutic efficacy. Therapeutic targets such as the HER-2/neu oncogene product can also be identified on circulating tumor cells (72). The availability of a standardized, reliable blood test could enable implementation of circulating tumor cells as a surrogate marker for clinical development of new anticancer agents and optimization of existing treatment protocols.

**References**


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