Detection and Clinical Importance of Micrometastatic Disease

Klaus Pantel, Richard J. Cote, Øystein Fodstad

Metastatic relapse in patients with solid tumors is caused by systemic preoperative or perioperative dissemination of tumor cells. The presence of individual tumor cells in bone marrow and in peripheral blood can be detected by immunologic or molecular methods and is being regarded increasingly as a clinically relevant prognostic factor. Because the goal of adjuvant therapy is the eradication of occult micrometastatic tumor cells before metastatic disease becomes clinically evident, the early detection of micrometastases could identify the patients who are most (and least) likely to benefit from adjuvant therapy. In addition, more sensitive methods for detecting such cells should increase knowledge about the biologic mechanisms of metastasis and improve the diagnosis and treatment of micrometastatic disease. In contrast to solid metastatic tumors, micrometastatic tumor cells are appropriate targets for intravenously applied agents because macromolecules and immunocompetent effector cells should have access to the tumor cells. Because the majority of micrometastatic tumor cells may be nonproliferative (G₀ phase), standard cytotoxic chemotherapies aimed at proliferating cells may be less effective, which might explain, in part, the failure of chemotherapy. Thus, adjuvant therapies that are aimed at dividing and quiescent cells, such as antibody-based therapies, are of considerable interest. From a literature search that used the databases MEDLINE®, CANCERLIT®, Biosis®, Embase®, and SciSearch®, we discuss the current state of research on minimal residual cancer in patients with epithelial tumors and the diagnostic and clinical implications of these findings. [J Natl Cancer Inst 1999;91:1113–24]

Malignant tumors of epithelial tissues are the most common form of cancer and are responsible for the majority of cancer-related deaths in Western industrialized countries. Because of progress in the surgical treatment of these tumors, mortality is linked increasingly to early metastasis, which is often occult at the time of primary diagnosis (1). For patients with no evidence of systemic metastases when the primary tumor is resected, traditional staging parameters of the tumor (e.g., tumor size and lymph node status) are determined; with this information and a statistical assessment of the risk of disease recurrence, the decision is made as to whether to give systemic adjuvant therapy to prevent metastatic relapse. Undetected micrometastases can contribute to the failure of primary treatment. Therefore, the identification of occult metastases in patients with early stage cancer could have a substantial clinical impact on the prognosis and optimal therapy for patients with cancer. For this reason, improved direct identification of minimal residual cancer is particularly important. At later stages of the disease, it may be useful to determine the presence of and change in the number of residual malignant cells so that the therapies selected can be monitored and adjusted to the changing needs of the patient.

Research into the molecular basis of tumor metastasis has identified numerous proteins that influence this process (Fig. 1). Conditions that allow growth of epithelial cells at metastatic sites are largely unknown but undoubtedly include the appropriate microenvironment for tumor cell growth (e.g., hormonal milieu, oxygenation, nutrients, or growth factors) and an environment for the formation of new blood vessels (angiogenesis). The factors determining the length of the period from the dissemination of tumor cells until the appearance of clinically manifest metastases are also unclear. From analyses of single cells, most disseminated tumor cells in bone marrow do not appear to be proliferating at the time of primary surgery (2,3). For this reason, it may be important to use adjuvant therapies that are aimed at both proliferating and nonproliferating cells.

This review was based on a literature search that used the databases MEDLINE®, CANCERLIT®, Biosis®, Embase®, and SciSearch®. We discuss the use of immunologic and molecular analyses in the diagnosis and characterization of minimal residual cancer. Available methods give access to this critical stage of tumor progression and can lead to the development of new therapeutic approaches that are aimed at preventing manifest metastasis.

DIAGNOSIS AND PROGNOSTIC RELEVANCE

Tumor Cell Dissemination by the Circulatory System

Although micrometastatic tumor cell aggregates may be identified by conventional histopathologic methods (4), individual disseminated carcinoma cells in bone marrow have generally resisted clear cytologic identification (5). In fact, the standard method rarely detects such cells in the bone marrow of patients with early stage operable cancer (6,7). During the past decade, more sensitive immunologic and molecular procedures have been developed that permit the identification of individual tumor cells in organs remote from the primary tumor (1,8). For epithelial tumors that tend to have skeletal metastases, individual tumor cells are easily detected among bone marrow cells aspirated from the iliac crest. The medullary space of the iliac crest...
Metastasis of Solid Tumors

Fig. 1. Diagram of tumor cell metastasis. Cells from the primary tumor leave the tumor as a result of proteolysis, infiltrate and invade the circulatory system, and migrate to a new site where they adhere to the walls of the capillary and invade a new organ. At this site, micrometastases (i.e., isolated tumor cells or small clusters of tumor cells) that are undetectable by conventional tumor staging procedures can survive for several years. The cells can evade chemotherapy because they are in a dormant state. We propose that this stage be called “M1.” Later, the cells become proliferative, stimulate angiogenesis, and begin to form a metastatic tumor (stage M2, tumor–lymph node–metastasis [TNM] classification system). † = Apoptotic tumor cells.

Table 1. Incidence of CK18-positive cells in bone marrow: tumor histology and clinical metastasis status

<table>
<thead>
<tr>
<th>Tumor stage†</th>
<th>No. of patients examined</th>
<th>No. of patients with CK18-positive cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammary carcinoma (two-sided P&lt;.001, χ² test)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M₀</td>
<td>116</td>
<td>35 (30.2)</td>
</tr>
<tr>
<td>M₁</td>
<td>19</td>
<td>14 (73.7)</td>
</tr>
<tr>
<td>Gastric carcinoma (two-sided P = .02, χ² test)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M₀</td>
<td>80</td>
<td>25 (31.3)</td>
</tr>
<tr>
<td>M₁</td>
<td>40</td>
<td>21 (52.5)</td>
</tr>
<tr>
<td>Colorectal carcinoma (two-sided P = .05, χ² test)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M₀</td>
<td>195</td>
<td>53 (27.2)</td>
</tr>
<tr>
<td>M₁</td>
<td>82</td>
<td>32 (39.0)</td>
</tr>
</tbody>
</table>

*CK = cytokeratin. CK18-positive cells were identified with monoclonal antibody CK2 (alkaline phosphatase anti-alkaline phosphatase-staining technique). Data are from (177).
†Tumor–lymph node–metastasis (TNM) classification system.

is a site of a particularly intensive exchange of cells between blood and the mesenchymal interstitium. Occult tumor cells are even detected in the bone marrow of patients who have cancers that generally do not metastasize to the bone (e.g., colon cancer), indicating that the bone marrow is a particularly good site for the detection of occult tumor cells. In contrast, detection of occult tumor cells in the peripheral blood of patients with early stage cancer is much more difficult because of the low frequency of these cells, and the clinical relevance of circulating tumor cells remains questionable (9–12). Thus, blood may be a suboptimal source for the detection of occult tumor cells (despite its obvious ease of sampling). In the future, it may be possible to enrich the tumor cell population in blood, which would improve its utility.

Detection of occult epithelial tumor cells in the bone marrow relies on methods that distinguish cells with different origins (e.g., hematopoietic cells versus epithelial cells), a concept introduced by investigators at the Royal Marsden Hospital and the Ludwig Institute nearly 20 years ago (13). Currently, most studies (5–7,13–22) that show an association between the success of a cancer patient’s treatment and the presence of occult micrometastases have used immunocytochemical methods (specific monoclonal antibodies) to detect extrinsic epithelial tumor cells. Several studies (17,21,22) have also shown that detection of occult tumor cells with monoclonal antibodies is clinically useful. However, caution needs to be exercised in the choice of marker antigens used; some marker antigens, such as epithelial membrane antigen or mucin-1, may not be suited for routine use because they also appear to be expressed on a subset of hematopoietic cells. Many studies (23,24) have used cytokeratins (CKs) as marker antigens; these proteins are stably and abundantly expressed in a majority of epithelial tumors and in a majority of the cells in these tumors. Although ectopic or illegitimate CK messenger RNA (mRNA) expression cannot be ruled out (25–29), many studies (16,30–33) have now shown that CK antigens are rarely detected in hematopoietic cells. Finally, a combination of several antibodies to various CK antigens or a broad spectrum of anti-CK antibodies has been used because of the antigenic heterogeneity of tumor cells (7,30,31,33).

When an antibody against CK18 was used as a probe, individual epithelial cells were found in the bone marrow aspirates from 20% to 30% of the patients examined who had primary carcinomas at different stages but no evidence of metastases (stage M₀, tumor–lymph node–metastasis [TNM] classification system; Table 1). However, most of the patients had fewer than 10 CK18-positive tumor cells per 8 × 10⁵ mononuclear bone marrow cells. Thus, techniques for detecting occult tumor cells must be extremely sensitive, well beyond the limits of sensitivity of standard histopathologic analysis. Immunocytochemical methods are exquisitely sensitive and can detect as few as one to two tumor cells in 1 × 10⁶ bone marrow mononuclear cells (30,33,34). Whether this level of sensitivity is adequate remains to be determined, but enrichment methods are now available that can increase the sensitivity by at least one order of magnitude (20,35–38).

When bone marrow aspirates from patients who had tumors in various stages were analyzed immunocytochemically (Table 1), the rate of early dissemination of tumor cells was similar for different types of carcinoma. When mammary carcinoma and colorectal carcinoma were compared, equivalent incidences of positive findings from both types of tumor were found in the bone marrow until the more advanced
stage M1, when the incidence of finding mammary carcinoma cells in the bone marrow was statistically significantly higher than the incidence of finding colorectal carcinoma cells (Table 1). The appearance of this difference at such an advanced tumor stage might be explained by a specific growth and/or survival advantage for the disseminated mammary carcinoma cells in bone marrow, in agreement with the “seed and soil” theory that Paget (39) proposed in 1889 [see also (40)] and with the presence of microenvironmental factors that support the growth of mammary tumor cells (41–43) but not the growth of colorectal tumor cells. This hypothesis could explain why clinically manifest skeletal metastasis from colorectal carcinoma is rare, despite the presence of disseminated colorectal tumor cells in bone marrow (44–47). Besides the interaction of tumor cells with the surrounding organ milieu, the path that cells must follow in the circulatory system also plays a role in tumor dissemination. Colon carcinoma cells, for example, must first pass through the capillary bed of the liver, which traps many of the cells and enhances the chances of metastasis of colon carcinoma to the liver (44).

The prognostic importance of the immunocytochemical identification of occult tumor cells in bone marrow has been confirmed in various prospective clinical studies (Table 2), several of which have shown that the presence of occult tumor cells in bone marrow is an independent risk factor (6,7,16,17,21,47–49). Because this is true even for tumors that rarely produce clinically important skeletal metastases, the appearance of epithelial cells in the bone marrow of patients with early stage cancer might indicate that tumor cells have also spread to other secondary sites, including the site of final metastatic deposit. However, not all studies have confirmed the prognostic relevance of occult tumor cell detection (50). It is possible that certain technical factors may account for these discrepancies. For early stage breast cancer, occult tumor cell detection rates of 4%–45% have been reported (18). The choice of antibodies and immunologic methods and the technical skills involved in performing the procedures and interpreting the results may introduce variation, and there is a certain risk of false-positive identification of nonepithelial cells (51–53). For example, prostate-specific antigen (PSA)-positive cells in the blood could be monocytes that have pinocytosed free PSA in serum or phagocytosed PSA-positive tumor cells (51,52). Thus, it will be important to define the critical variables in the methods and to introduce at least some level of standardization to allow more reliable and reproducible results (30,33,53). Finally, differences in study design may account for some of the discrepancies; some studies have not been designed to allow evaluation of the association of occult tumor detection with clinical outcome.

In addition to the presence or absence of occult tumor cells, it now appears that the number of tumor cells detected may be clinically relevant. Preliminary work by Cote et al. (6) indicates that there is an increased risk of relapse for patients with mammary carcinoma who have more disseminated cells. By quantitatively transferring cells to slides by cytocentrifugation, Jauch et al. (48) showed that the occult tumor cell count is associated with the rate of relapse in patients with gastric carcinoma. Thus, the number of occult disseminated tumor cells detected may reflect the tumor cell burden, and this number may be an important clinical variable. This result again highlights the importance of reliable and reproducible techniques to detect such cells.

In studies of patients who had either prostate or colorectal carcinoma or who had melanoma, peripherally obtained peripheral blood samples were examined by molecular methods (see below). Results showed that a temporary intraoperative dissemination of tumor cells into circulation can occur (54–57). It is not known whether these cells reach, survive, and form detectable metastases in secondary organs.

### Tumor Cell Dissemination Through Lymph Nodes

The presence or absence of metastases to regional lymph nodes is the single most important standard risk factor for patients with the most solid tumors when no evidence of systemic metastases is present. However, routine histopathologic examination of lymph nodes will underestimate the prevalence of such metastases; in fact, it has been calculated that a pathologist has only a 1% chance of identifying a small (three-cell diameters) metastatic focus of cancer (38).

The detection of occult metastases in the lymph nodes of patients with node-negative cancer is being shown to be prognostically important in an increasing number of studies on many types of cancers, including breast cancer (59–62), colon cancer (63,64), gastric cancer (65), non-small-cell lung cancer (66–68), esophageal cancer (69), prostate cancer (70,71), and melanoma (72). These results emphasize the importance of verifying the lymph node status, which may improve tumor staging and may provide additional criteria for administering adjuvant therapy. Although it seems obvious that regional tumor spread is clinically important, several investigations [reviewed in (73–75)] have found that such tumor deposits are not associated with clinical outcome. This appears to be largely the result of study design; most of the negative studies involved too few patients to address the issue with sufficient statistical power. In addition, technical issues may account for discrepancies in some studies. For example, the analysis of a few sections, 5–6 μm thick, represents a relatively small random sample of a lymph node. Furthermore, in general, the use of anti-CK antibodies appears to be a reliable and an effective method for tumor cell detection, although normal lymph node (reticulum) cells can express CKs (e.g., CK19) (23). However, antibodies against other epithelial

### Table 2. Immunocytochemical studies of the prognostic relevance of disseminated tumor cells in bone marrow

<table>
<thead>
<tr>
<th>Type of tumor</th>
<th>Marker protein(s)</th>
<th>Detection rate (%)</th>
<th>Prognostic value</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammary carcinoma</td>
<td>EMA</td>
<td>89/350 (25)</td>
<td>DFS, OS</td>
<td>(22)</td>
</tr>
<tr>
<td></td>
<td>EMA, TAG12, CK</td>
<td>38/100 (38)</td>
<td>DFS, OS†</td>
<td>(21)</td>
</tr>
<tr>
<td></td>
<td>CK</td>
<td>18/48 (37)</td>
<td>DFS†</td>
<td>(6)</td>
</tr>
<tr>
<td></td>
<td>TAG12</td>
<td>315/727 (43)</td>
<td>DFS, OS†</td>
<td>(17)</td>
</tr>
<tr>
<td>Colorectal carcinoma</td>
<td>CK18</td>
<td>28/88 (32)</td>
<td>DFS†</td>
<td>(47)</td>
</tr>
<tr>
<td>Gastric carcinoma</td>
<td>CK18</td>
<td>34/97 (35)</td>
<td>DFS</td>
<td>(175)</td>
</tr>
<tr>
<td></td>
<td>CK18</td>
<td>47/78 (60)</td>
<td>DFS</td>
<td>(141)</td>
</tr>
<tr>
<td></td>
<td>CK18</td>
<td>95/180 (53)</td>
<td>DFS</td>
<td>(48)</td>
</tr>
<tr>
<td>Esophageus carcinoma</td>
<td>CK</td>
<td>37/90 (41)</td>
<td>DFS, OS</td>
<td>(176)</td>
</tr>
<tr>
<td>Bronchial carcinoma</td>
<td>CK</td>
<td>17/43 (40)</td>
<td>DFS</td>
<td>(7)</td>
</tr>
<tr>
<td>NSCLC*</td>
<td>CK18</td>
<td>83/139 (60)</td>
<td>DFS†</td>
<td>(16)</td>
</tr>
</tbody>
</table>

*EMA = epithelial membrane antigen; CK = cytokeratin; TAG12 = tumor-associated glycoprotein 12; DFS = disease-free survival; OS = overall survival; and NSCLC = non-small-cell lung cancer.

†Prognostic value as an independent parameter was confirmed through multivariate analysis.
antigens that are not present on normal lymph node cells have been used, including BerEP4, an antibody that recognizes two glycoproteins of 34 and 49 kd present on the cell surface (66,67,69), and an antibody against carcinoembryonic antigen (CEA) (76).

In addition to immunohistochemistry, molecular methods based on the polymerase chain reaction (PCR)-mediated amplification of tumor cell DNA or of complementary DNA reverse transcribed from mRNA have been used to detect tumor cells in lymph nodes. However, the specificity of RNA-based markers, such as CEA mRNA, recently used for the analysis of lymph nodes, is not absolute because of the low-level illegitimate expression of the marker gene in the surrounding lymph node cells (77). Better alternatives are DNA-based markers, such as mutations in the p53 gene or Ki-ras gene, that have been used in patients with colorectal cancer, lung cancer, or head and neck cancer to detect single tumor cells in a background of thousands of lymph node cells (78–80).

An important advance in the evaluation of regional lymph nodes has been the development of a more limited dissection, the sentinel lymph node dissection, that is based on the identification, with dyes or radioactivity, of the specific lymph node that drains the tumor and the removal of this lymph node for analysis. This approach was pioneered by Morton et al. (81) and Giuliano et al. (82,83) and has been extensively evaluated in patients with melanoma and breast cancer (81–85). Although the advantages of a more limited lymph node dissection are clear (in particular, the potential for decreasing the rate of postoperative complications), there is less material available for staging evaluation. The use of sensitive methods to detect micrometastasis may allow the identification of metastases in more limited amounts of material, such as lymph nodes, and thus may influence the subsequent therapeutic approach (e.g., more extensive lymph node dissection and the administration of adjuvant therapy). The use of immunohistochemistry can change the status of a negative lymph node to a positive lymph node in 5–20% of the sample tested, and thus inclusion of an immunohistochemical evaluation may reduce the false-negative rate of the sentinel lymph node technique to almost zero (86). Thus, detection of occult tumor cells may be an important adjunct to the use of limited lymph node dissection for staging and for therapy (87,88).

**NEW TECHNIQUES FOR DETECTION OF DISSEMINATED TUMOR CELLS**

The immunocytocchemical detection of micrometastases has been developed during the last 10 years, and its clinical relevance has been validated. This method is currently the standard method for the early detection of occult tumor cells disseminated from solid tumors. Microscopic analysis of many cytologic samples is, however, time consuming and requires considerable expertise. A new method of cytocentrifugation (Hettich, Tuttingen, Germany) that permits the analysis of larger sample volumes (89) should address the first problem. The microscopic screening of large numbers of cytologic samples could be automated by the use of an image-analysis system (scanner). Systems of this type are currently being developed that have high sensitivity and specificity and can be used for screening occult metastases in patients enrolled in clinical trials (90,91). One way to increase the sensitivity of tumor cell detection in bone marrow and blood is to selectively enrich for tumor cells. This enrich-
population of cells (8). However, the tumor cell must have specific changes in its genome or mRNA expression pattern that distinguish it from the surrounding hematopoietic cells. At the DNA level, this criterion is difficult for most solid tumors to meet because the cells are quite genetically heterogeneous. Screening for genomic changes requires the molecular analysis of the primary tumor from each patient to determine the individual genomic alterations of that tumor. Exceptions are colon and pancreatic carcinomas, which commonly harbor distinct mutations of the Ki-ras oncogene that has been targeted for the detection of occult tumor cells in lymph nodes (78,103), blood (104,105), bone marrow (106), and liver (107). In tumors with a virus-associated oncogenesis, such as cervical cancer, screening of lymph nodes for human papillomavirus DNA and mRNA may be a fruitful approach (108). In light of earlier studies (109–112) indicating that cancer patients have larger amounts of circulating DNA in serum or plasma, blood samples from patients with head and neck tumors or lung cancer have been analyzed for microsatellite alterations (113,114). With the rapid advancement of new technologies that allow the profiling of individual tumors (115), the development of methods for patient-specific tumor cell detection may be possible. Another interesting application of DNA-based markers is the analysis of the p53 [also known as TP53] gene in cells of the resection margins, which are called tumor free by conventional histopathologic examination. This type of analysis has been shown to provide clinically important data for patients with squamous cell carcinomas of the head and neck (80).

The detection of differentially expressed mRNA species, on the other hand, appears to present fewer obstacles to more widespread use of the PCR method (73), in which the cell’s mRNAs are transcribed into complementary DNAs by reverse transcription (RT), and the complementary DNAs are amplified in a subsequent PCR (RT–PCR). In this method, mRNAs that are differentially expressed in epithelial cells (i.e., occult tumor) compared with hematopoietic cells are amplified, exactly analogous to the immunohistochemical detection of occult tumor cells with antibodies specific for epithelial cell antigens. Because CKs are highly expressed in epithelial tumors, they have frequently been targets, particularly CK19 and CK20 (9,116–120), although many epithelial markers have been evaluated (Table 3). Other transcripts used as markers include CEA (64,121), epidermal growth factor receptor (122,123), mucin-1 (119), human chorionic gonadotropin-β (124), and α-fetoprotein (125). In prostate cancer, prostate-specific marker transcripts are available, including PSA, prostate-specific membrane antigen, and human kallikrein-2 [reviewed in (73)]. However, it is now becoming clear that many of these targets may not have the requisite specificity to distinguish epithelial tumor cells from hematopoietic cells. Several studies (25–29,118,126–129) have shown that these transcripts are consistently identified in normal bone marrow, blood, and lymph node tissue. There are several possible reasons for this lack of specificity, including the presence of pseudogenes and low-level transcription of epithelium-specific mRNA by hematopoietic cells. Besides the choice of the appropriate marker transcript, the specificity of the RT–PCR assay largely depends on the method of sample preparation and on the assay conditions (73,130,131). For example, false-positive findings of RT–PCR assays for CK20 can be avoided by analysis of mononuclear cells instead of whole blood preparations, because normal granulocytes express CK20 (9,132). Another limiting factor is the deficient expression of the marker gene (e.g., PSA) in micrometastatic tumor cells (27,133).

### Table 3. Detection of disseminated epithelial tumor cells by molecular methods

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Tumor organ</th>
<th>Messenger RNA/DNA marker(s)* (reference Nos.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow</td>
<td>Breast</td>
<td>CK19, CEA (116,121)</td>
</tr>
<tr>
<td></td>
<td>Colorectum</td>
<td>Ki-ras mutations, CEA, CK19, CK20 (9,106,118,121)</td>
</tr>
<tr>
<td></td>
<td>Stomach</td>
<td>CEA, CK20 (9,121)</td>
</tr>
<tr>
<td></td>
<td>Pancreas</td>
<td>CEA (121)</td>
</tr>
<tr>
<td></td>
<td>Prostate</td>
<td>PSA, CK19, hK2 (73)</td>
</tr>
<tr>
<td></td>
<td>Head and neck</td>
<td>E48 antigen (178)</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>Breast</td>
<td>CK19, MUC1, β-hCG (119,120,124)</td>
</tr>
<tr>
<td></td>
<td>Lung</td>
<td>p53, Ki-ras mutations (79)</td>
</tr>
<tr>
<td></td>
<td>Cervix</td>
<td>HPV16, E6/E7 (108)</td>
</tr>
<tr>
<td></td>
<td>Colorectum</td>
<td>p53 and Ki-ras mutations, CEA, CK19, CK20 (64,78,118)</td>
</tr>
<tr>
<td></td>
<td>Prostate</td>
<td>PSA, PSM, hK2 (73)</td>
</tr>
<tr>
<td></td>
<td>Head and neck</td>
<td>p53 mutations (80)</td>
</tr>
<tr>
<td>Blood</td>
<td>Breast</td>
<td>CK19, EGF receptor, β-hCG (122–124)</td>
</tr>
<tr>
<td></td>
<td>Lung</td>
<td>Microsatellite alterations (114)</td>
</tr>
<tr>
<td></td>
<td>Colorectum</td>
<td>CK20, Ki-ras mutations (9,104,117)</td>
</tr>
<tr>
<td></td>
<td>Stomach</td>
<td>CK20 (9)</td>
</tr>
<tr>
<td></td>
<td>Pancreas</td>
<td>Ki-ras mutations (105)</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>α-Fetoprotein (125)</td>
</tr>
<tr>
<td></td>
<td>Prostate</td>
<td>PSA, PSM, hK2 (73)</td>
</tr>
<tr>
<td></td>
<td>Head and neck</td>
<td>Microsatellite alterations (113)</td>
</tr>
<tr>
<td></td>
<td>Pancreas</td>
<td>Ki-ras mutations (107)</td>
</tr>
<tr>
<td>Liver</td>
<td>Head and neck</td>
<td>p53 mutations (80)</td>
</tr>
</tbody>
</table>

*CK = cytokeratin; CEA = carcinoembryonic antigen; PSA = prostate-specific antigen; MUC = mucin; HPV = human papillomavirus; EGF = epidermal growth factor; PSM = prostate-specific membrane antigen; β-hCG = β subunit of human chorionic gonadotropin; and hK2 = human kallikrein-2.

### Biologic Characteristics of Disseminated Cancer Cells

The detection of disseminated tumor cells has introduced a new opportunity to evaluate which of the diverse biologic characteristics of the primary tumor might favor the early dissemination of its cells. Two groups (136,137) have recently reported an association of tumor angiogenesis with bone marrow micrometastases for breast and gastric cancers. In addition, Choy and McCulloch (138) and McCulloch et al. (139) found an association between tumor angiogenesis and tumor cell shedding into
effluent venous blood during breast cancer surgery. The metastatic potential to bone marrow was not associated with the expression of p53 and RB genes or the proliferative activity of the primary lesion of gastric cancer (140). In view of the malignant potential of CK-positive cells, a number of tumor-associated characteristics have been identified in CK-positive cells with immunocytochemical double-staining methods, including expression of urokinase plasminogen activator receptor, overexpression of the erbB2 oncogene, and deficient expression of major histocompatibility complex (MHC) class I molecules (Table 4).

Urokinase plasminogen activator receptor expression on disseminated tumor cells in bone marrow of patients with gastric cancer was associated with increasing tumor cell counts and poor clinical prognosis (141,142). This finding suggests that expression of the urokinase plasminogen activator receptor not only is involved in tumor invasion but also influences the survival and/or growth of disseminated tumor cells in bone marrow, which is consistent with the currently accepted role of proteinases in metastasis (143). Another selection criterion for tumor cell dissemination might be overexpression of the erbB2 oncogene, which was frequently observed on bone marrow micrometastases (2,3). It is interesting that patients with breast cancer exhibited distinctly higher incidences of p185erbB2 expression on micrometastases (60%–70%) compared with their primary tumors (20%–30%), indicating that erbB2 overexpression might be a positive selection criterion for disseminated tumor cells. All breast carcinoma patients analyzed who had distant metastases (stage M1) had p185erbB2 on CK-positive cells compared with about 50% of the patients who had regional disease (stage M0; TNM classification system). More recently, Brandt et al. (144) suggested that blood-borne c-erbB2-positive CK-positive clustered cells are the possible precursors of distant metastases. These findings might explain why antibody therapy directed against erbB2-expressing cancer cells appears to be successful in patients with metastatic breast cancer who are receiving additional chemotherapy (145,146).

The low frequency of epithelial tumor cells in bone marrow and the localization of epithelial cells in an organ that has such a good blood supply offer ideal conditions for the elimination of epithelial cells by immunocompetent cells. The clinical history of epithelial tumor cells shows, however, that micrometastatic tumor cells can be ignored for many years by the immune system. In this context, the deficient expression of MHC class I molecules (147,148), which, as restrictive elements, participate in T-lymphocyte-mediated tumor cell recognition, may be an important survival feature (Table 4). The underexpression of MHC class I molecules could limit the prospects for success of tumor cell vaccines (149). Antibody-mediated tumor cell killing, on the other hand, is independent of tumor-cell MHC expression.

The malignant nature of CK-positive cells in bone marrow has been further confirmed through genomic analysis by the fluorescence in situ hybridization, in which many aberrations in chromosomes 7, 8, and 18 and the amplification of the erbB2 gene were observed in these cells (150,151). The sensitivity of this procedure for detecting cells with amplified erbB2 and Int2 genes was increased by including an immunomagnetic enrichment step for CK-positive cells before the test (152). Extensive cell culture experiments have also shown that cells disseminating into the bone marrow have a time-limited proliferative potential (153). Thus, these cells apparently cannot yet proliferate autonomously and may be dormant (154). This assumption has been corroborated by double-staining studies (2,3), in which the fraction of disseminated tumor cells in bone marrow that express a proliferation marker (Ki-67 or p120) appears to be small. The dormant state of these cells may be one explanation of the relative resistance of micrometastatic tumor cells to chemotherapy and would confirm the appropriateness of therapies that are independent of the proliferative status of the cells targeted. Multiple labeling experiments [e.g., using antibody-coupled fluorescent particles (12)] may allow various tumor cell characteristics to be ranked according to their utility as therapeutic targets. Furthermore, cell lines established from bone marrow micrometastases of cancer patients are now available to evaluate new anticancer agents (155,156).

**Therapy**

Adjuvant therapy for patients with early stage operable disease has been shown to be an important component in the management of many cancers. Conventional adjuvant chemotherapy has been modified and improved in various ways (157,158). For colorectal cancer, it has been shown that adjuvant chemotherapy is effective in some patients and generally well tolerated (159); the efficacy threshold in chemotherapy protocols published to date is an approximately 30% reduction in mortality (159,160). The success of adjuvant therapy is assumed to stem from its ability to eradicate occult metastases before they become clini-

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**Table 4. Phenotype of cytokeratin (CK)-positive tumor cells in bone marrow**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Tumor origin</th>
<th>Marker-positive or CK-positive cells.*</th>
<th>No. of patients with marker/No. of total patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth factor receptor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>erbB2</td>
<td>Breast</td>
<td>48/71 (67.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Colorectum</td>
<td>8/28 (28.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stomach</td>
<td>6/22 (27.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lung</td>
<td>5/6 (83.3)</td>
<td></td>
</tr>
<tr>
<td>Transferrin receptor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Breast</td>
<td>17/59 (28.8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Colorectum</td>
<td>7/17 (41.1)</td>
<td></td>
</tr>
<tr>
<td>MHC class I antigen†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Breast</td>
<td>9/26 (34.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Colorectum</td>
<td>12/17 (70.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stomach</td>
<td>8/11 (72.7)</td>
<td></td>
</tr>
<tr>
<td>Adhesion molecule</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17-1A (EpCAM)†</td>
<td>Breast</td>
<td>20/31 (64.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lung (NSCLC†)</td>
<td>13/31 (41.9)</td>
<td></td>
</tr>
<tr>
<td>Plakoglobin</td>
<td>Lung (NSCLC†)</td>
<td>4/12 (33.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Colorectum</td>
<td>4/13 (30.8)</td>
<td></td>
</tr>
<tr>
<td>Proliferation-associated protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ki-67</td>
<td>Breast</td>
<td>1/12 (8.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Colorectum</td>
<td>0/13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stomach</td>
<td>0/8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lung</td>
<td>0/7</td>
<td></td>
</tr>
<tr>
<td>p120</td>
<td>Breast</td>
<td>1/11 (9.1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Colorectum</td>
<td>5/12 (41.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stomach</td>
<td>4/13 (30.8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lung</td>
<td>3/10 (30)</td>
<td></td>
</tr>
<tr>
<td>Protease uPA† receptor</td>
<td>Stomach</td>
<td>20/44 (45)</td>
<td></td>
</tr>
</tbody>
</table>

*From (2,3,141,142,147,148,179,180).†uPA = urokinase plasminogen activator; MHC = major histocompatibility complex; ICAM-1 = intercellular adhesion molecule-1; NSCLC = non-small-cell lung cancer; and EpCAM = epithelial cell adhesion molecule.

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cally evident (161). However, the success of standard adjuvant chemotherapy, particularly chemotherapy aimed at proliferating cell populations, may be limited by the fact that many of the residual systemic tumor cells present after primary resection may be nonproliferative or dormant (2,3).

The basic idea proposed by Paul Ehrlich of treating tumors with specific antibodies (“magic bullets”) is more than 100 years old. The hybridoma technique for making monoclonal antibodies in large quantities (162) was described in 1975 and has presented a vast array of potential therapeutic options (i.e., specific targets expressed by the cancer cells). Although monoclonal antibody therapy has been effective in various experimental systems, the clinical experience has been disappointing for patients with advanced stage solid tumors [reviewed in (1,146)], probably as a result of the large tumor cell burden and the lack of access that macromolecules have to cells in large tumors (163). Complete remission has nevertheless been induced in some patients with metastatic colorectal carcinoma through a combination therapy for monoclonal antibody 17-1A and granulocyte–macrophage colony-stimulating factor (164). Another approach is the use of antibody–toxin conjugates, or immunotoxins (165,166), and promising effects with such agents, even in advanced disease, have been reported (167).

Despite these results, however, micrometastatic or isolated tumor cells should in theory be much more promising targets for antibody-based therapy (1,146). The incidence of micrometastatic cells in mesenchymal tissue such as bone marrow makes them easily accessible for intravenously applied macromolecules, a vital prerequisite for the effectiveness of these forms of therapy. For free antibodies, this therapeutic rationale has been examined in a randomized study of patients with colorectal carcinoma of International Union Against Cancer stage III after complete resection of the primary tumor. The patients were given five postoperative infusions of the monoclonal antibody edrecolomab against the 17-1A antigen (PanorexR; GlaxoWellcome, Hamburg, Germany) as adjuvant therapy. During a 7-year period, the test group showed a substantial reduction in mortality and, in particular, a reduction in remote metastasis formation compared with the control group (168).

The heterogeneity of solid tumors poses a problem for all types of therapy and limits the chances of complete elimination of all residual tumor cells. Although expression of the 17-1A antigen is relatively homogeneous in colon carcinoma cells, it is more heterogeneous in disseminated mammary carcinoma cells (Table 4). This highlights the value of characterizing the micrometastatic cells in individual patients before antibody-based therapy is initiated. Multiple analyses of tumor cells isolated from bone marrow or peripheral blood could characterize the cells and guide the choice of antibody and/or conjugate for individual patients. Because of the heterogeneity of the residual carcinoma cells (Table 4), it might also be possible to use a mixture of antibodies and/or immunotoxins that target different membrane proteins expressed by the tumor cells to achieve the greatest possible therapeutic effect.

Occult tumor cells in the bone marrow of patients with early stage cancer have been the target of another class of therapy, where it has recently been shown that the bisphosphonate clodronate (OstacR; Boehringer Mannheim GmbH, Mannheim, Germany) can reduce the incidence of developing overt metastases in patients with early stage breast cancer who have occult metastases detected in their bone marrow (169). Thus, this therapy aimed at a population of patients at risk for developing metastases (i.e., those with occult systemic disease at the time of presentation) has been shown to be beneficial.

**CONCLUSIONS AND OUTLOOK**

Despite the progress made in clinical oncology in recent decades, the presence of minimal residual cancer has limited the prospects for further improvements in lethality rates.

Although conventional tumor-staging parameters can provide reliable information about the proportion of a population of patients who will experience a recurrence of the disease, these measures cannot predict which individuals will have a recurrence of disease after primary therapy, particularly if the patient has early stage disease. Thus, new parameters need to be defined that better identify those patients at the greatest (and at the least) risk of relapse, because this would provide information critical to the subsequent management of the patient. The detection of the earliest manifestations of tumor dissemination is an extremely promising approach that should improve risk assessment and the identification of specific patients who would benefit from adjuvant treatment. During the last 10 years, new immunologic and molecular analytic procedures have been developed to diagnose and characterize minimal residual cancer. Studies are currently in progress to evaluate and standardize these procedures for clinical use. The encouraging results to date from studies on the prognostic relevance of disseminated tumor cells in bone marrow should be standardized, categorized, and incorporated into the staging nomenclature of the International Union Against Cancer. As part of the pathologic assessment process, additional tumor-staging information could be provided by including micrometastases in the TNM classification system (170). Improved methods for genomic analysis of single tumor cells (106,171,172) and for assessment of target molecule expression may increase the diagnostic precision of current detection techniques and optimize the therapy for individual patients.

As far as adjuvant therapy is concerned, success or failure can be assessed only after an observation period of several years. The availability of a surrogate marker for monitoring the effectiveness of a treatment should speed the evaluation and development of new adjuvant therapies. Periodic examination of bone marrow and peripheral blood during therapy could indicate whether the therapeutic approach being used was effective. Monitoring procedures of this type would be of considerable value. Because of their accessibility, bone marrow or peripheral blood samples would be logical contenders for monitoring minimal residual cancer at the subclinical stage. Our experience to date indicates that immunologic or molecular monitoring of disseminated cells is in principle possible for individual patients and that a step involving the reproducible enrichment of rare tumor cells for these tests would be desirable to improve the chances of detecting tumor cells over the course of longitudinal studies (173,174). In therapeutic studies, long-term observations are still required to establish whether the therapy-associated reduction in individual disseminated cells is associated with improved prognosis. In conclusion, we believe that there is an increasing body of evidence demonstrating that detection and characterization of tumor cells disseminated in bone marrow or peripheral blood can provide clinically important data that are of value for tumor staging and for prognostication and that can identify surrogate markers for early assessment of the effectiveness of adjuvant therapy. Thus, these data would have a sub-
stancial influence on future oncologic diagnosis and treatment. At the very least, examination for occult metastases should be incorporated into future clinical trials to evaluate cancer treatment. In the future, adjuvant therapy, specifically tailored to the disease in subgroups of patients or individual patients with residual disease, may represent a substantial advance.

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NOTE

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