Rare Expression of Epithelial Cell Adhesion Molecule on Residual Micrometastatic Breast Cancer Cells after Adjuvant Chemotherapy

Holger Thurm, Sebastian Ebel, Christina Kentenich, Alice Henssen, Sabine Riethdorf, Cornelia Coith, Diethelm Wallwiener, Stephan Braun, Carsten Oberhoff, Fritz Jänicek, and Klaus Pantel, on behalf of the German Study Group of Clinical Trial MA 220002

Institute for Tumor Biology [H. T., A. H., S. R., C. C., K. P.] and Clinic for Gynecology [S. E., F. J.], University Hospital Hamburg-Eppendorf, D-20246 Hamburg, Germany; University Clinic for Obstetrics and Gynecology Tübingen, D-72076 Tübingen, Germany [D. W.]; University Clinic for Obstetrics and Gynecology Innsbruck, Innsbruck A-6020, Austria [S. B.]; Ludwig-Maximillian University Clinic for Obstetrics and Gynecology, D-80337 München, Germany [C. K.]; and Center for Gynecologic Oncology of the University Clinic Essen, D-45122 Essen, Germany [C. O.]

ABSTRACT

Purpose: Over the past 5 years, several clinical studies in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. 1 Supported by a grant of GlaxoWellcome, Hamburg, Germany, and the Small Study Group of Clinical Trial MA 220002. 2 To whom requests for reprints should be addressed, at Institut für Tumorbiologie, Universitätsklinikum Eppendorf, Martinistrasse 52, D-20251 Hamburg, Germany. Phone: 49-40-42803-5303; Fax: 49-40-42803-5379; E-mail: pantel@uke.uni-hamburg.de.

Received 2/12/02; revised 2/20/03; accepted 3/11/03. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by a grant of GlaxoWellcome, Hamburg, Germany, and the Deutsche Krebshilfe/Dr. Mildred Scheel Stiftung, Bonn, Germany.

2 To whom requests for reprints should be addressed, at Institut für Tumorbiologie, Universitätsklinikum Eppendorf, Martinistrasse 52, D-20251 Hamburg, Germany. Phone: 49-40-42803-5303; Fax: 49-40-42803-5379; E-mail: pantel@uke.uni-hamburg.de.

Other members of the German study group of clinical trial MA 220002: J. W. Goethe-Universität, Zentrum der Frauenheilkunde und Geburtshilfe, Theodor-Stern-Kai 7, 60590 Frankfurt (G. v. Minckwitz); Augusta-Kranken-Anstalt, Hämatologisch-Onkologische Ambulanz, Bergstraße 26, 44791 Bochum (K. Bremer); Gemeinschaftspraxis Dr. Bruderl/Dr. Heinrich, Halderstraße 29, 86150 Augsburg (B. Heinrich); Klinik Bad Trissl, Abteilung für Gynäkologie I, 83080 Oberaudorf (J. Bischoff); Gemeinschaftspraxis Dr. Springer/Dr. Fiechtner, Königstraße 1B, 70173 Stuttgart (H. Fiechtner); Universitätsklinikum der Humboldt-Universität zu Berlin, Klinik für Chirurgie und Chirurgische Onkologie, Lindenberger Weg 80, 13125 Berlin (P. Schlag); Praxis Dr. Maasberg, Siegfriedstraße 20, 56727 Mayen (M. Maasberg); Praxis Dr. Schließer/Dr. Kabisch, Wingertshöhe 6, 53592 Gießen (A. Kabisch); Praxis Dr. Dengler, Bahnhofstraße 24, 93047 Regensburg (R. Dengler); Lukas-Krankenhaus, Abteilung für Innere Medizin, Preußenstraße 84, 40401 Neuss (P. Czygan); Praxis Dr. Lohse, Hamner Landstraße 1a, 41480 Neuss (C. Lohse); Asklepios Klinik Lich, Frauenklinik, 35423 Lich (V. Kuhlner); Praxis Dr. Kemper, Hugo-Fuchs-Allee 3, 58644 Iserlohn (J. Kemper); Caritasinstitut/St. Theresa, Frauenklinik, Rheinstraße 2, 66113 Saarbrücken (K. Neis)

Received 2/12/02; revised 2/20/03; accepted 3/11/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. 1 Supported by a grant of GlaxoWellcome, Hamburg, Germany, and the Deutsche Krebshilfe/Dr. Mildred Scheel Stiftung, Bonn, Germany.

2 To whom requests for reprints should be addressed, at Institut für Tumorbiologie, Universitätsklinikum Eppendorf, Martinistrasse 52, D-20251 Hamburg, Germany. Phone: 49-40-42803-5303; Fax: 49-40-42803-5379; E-mail: pantel@uke.uni-hamburg.de.

Other members of the German study group of clinical trial MA 220002: J. W. Goethe-Universität, Zentrum der Frauenheilkunde und Geburtshilfe, Theodor-Stern-Kai 7, 60590 Frankfurt (G. v. Minckwitz); Augusta-Kranken-Anstalt, Hämatologisch-Onkologische Ambulanz, Bergstraße 26, 44791 Bochum (K. Bremer); Gemeinschaftspraxis Dr. Bruderl/Dr. Heinrich, Halderstraße 29, 86150 Augsburg (B. Heinrich); Klinik Bad Trissl, Abteilung für Gynäkologie I, 83080 Oberaudorf (J. Bischoff); Gemeinschaftspraxis Dr. Springer/Dr. Fiechtner, Königstraße 1B, 70173 Stuttgart (H. Fiechtner); Universitätsklinikum der Humboldt-Universität zu Berlin, Klinik für Chirurgie und Chirurgische Onkologie, Lindenberger Weg 80, 13125 Berlin (P. Schlag); Praxis Dr. Maasberg, Siegfriedstraße 20, 56727 Mayen (M. Maasberg); Praxis Dr. Schließer/Dr. Kabisch, Wingertshöhe 6, 53592 Gießen (A. Kabisch); Praxis Dr. Dengler, Bahnhofstraße 24, 93047 Regensburg (R. Dengler); Lukas-Krankenhaus, Abteilung für Innere Medizin, Preußenstraße 84, 40401 Neuss (P. Czygan); Praxis Dr. Lohse, Hamner Landstraße 1a, 41480 Neuss (C. Lohse); Asklepios Klinik Lich, Frauenklinik, 35423 Lich (V. Kuhlner); Praxis Dr. Kemper, Hugo-Fuchs-Allee 3, 58644 Iserlohn (J. Kemper); Caritasinstitut/St. Theresa, Frauenklinik, Rheinstraße 2, 66113 Saarbrücken (K. Neis)
adjuvant immunotherapy directed against micrometastatic disease (6). However, Ep-CAM might be down-regulated on metastatic tumor cells because loss of cell-cell adhesion is a prerequisite for tumor cell dissemination (3).

Systemic adjuvant therapy is generally aimed to eradicate disseminated tumor cells as the potential seed for subsequent metastatic relapse. Because of their epithelial origin, these cells can be identified in hematopoietic organs (blood and bone marrow) with monoclonal antibodies against CK, the epithelial specific intermediate filaments that are not expressed by hematopoietic cells. In a recent study on >500 breast cancer patients, we could demonstrate the specificity and independent prognostic significance of CK-positive cells in bone marrow (7). In addition, various studies published over the past 10 years have demonstrated the prognostic significance of immunoocytochemically identified cancer cells in the bone marrow of breast cancer patients (7–12).

A previous pilot study on a small cohort of mainly metastatic breast cancer patients revealed a heterogeneous expression pattern of Ep-CAM (13), and treatment of these patients with the Ep-CAM-specific monoclonal antibody Edrecolomab (Panorex) resulted in a selective reduction of Ep-CAM-positive tumor cells in bone marrow (14). These findings suggest a potential role of Ep-CAM-directed antibody therapy in patients with Ep-CAM-positive metastatic breast cancer cells.

Because chemotherapy and hormonal therapy still play the major role in the adjuvant treatment of primary breast cancer, antibody therapy with Edrecolomab can only be envisaged as a second-line treatment. Thus far, there is no information available regarding the status of Ep-CAM expression on occult metastatic breast cancer cells that survived first-line systemic chemotherapy. Our present results indicate that cancer cells in bone marrow can survive such chemotherapy, suggesting that some of these cells are unaffected or even resistant to the applied cytotoxic agents. However, the effect of an additional anti-Ep-CAM antibody therapy might be limited because these cells rarely express Ep-CAM.

**PATIENTS AND METHODS**

**Patients and Treatment.** In total, 62 breast cancer patients were admitted to different German hospitals and ambulant oncologists between October 1999 and October 2000 (see list of clinical contributors). All patients presented without distant metastases, and they had <10 tumor-positive lymph nodes (Table 1). Patients with nodal status >10 were excluded because we expected that these patients may already have hidden overt metastases and may therefore not represent patients with minimal residual disease. The primary surgical treatment consisted of either breast conservation or modified radical mastectomy, leading to R0 resection in all reported cases. In all patients treated with breast conservation, radiation therapy was administered (between 50 and 60 Gy). At the time of primary surgery, complete baseline diagnostic evaluation for distant metastases included plain chest radiography, (contralateral) mammography, ultrasonography of the liver, and whole body scan. In case of evidence for distant disease, patients were excluded from the study. Bone marrow samples were taken if patients were treated with at least two or more courses of various forms of adjuvant chemo and/or radiotherapy (Table 1) and/or not later than 3 months after completion of adjuvant therapy. All patients gave their informed consent on study entry, and the study protocol was approved by the Ethics Committee.

**Immunohistochemistry of Primary Tumors.** All samples were routinely formalin fixed and paraffin embedded. After deparaffinization, automatic immunostaining was performed on the DAKO Autostainer using the mouse antihuman epithelial-specific antigen monoclonal antibody (clone VU-1D9, concentration 1:100; Novocastra, Newcastle, United Kingdom) and the DAKO ChemMate (horseradish peroxidase; DAKO, Hamburg, Germany) Detection Kit. Application of the primary antibody was followed by incubation with biotinylated goat antimouse immunoglobulins, streptavidin conjugated to horseradish peroxidase, 3,3’-diaminobenzidine tetrahydrochlorid as chromogen, buffered solution containing hydrogen peroxide, and hematoxylin counterstaining. The breast cancer cell line MCF-7 (American Type Culture Collection HTB 22) was used as positive control. MOPC-21 (Sigma, Deisenhofen, Germany), an unrelated mouse myeloma immunoglobulin, served as the IgG1 isotype control at an appropriate dilution.

**Bone Marrow Preparation and Detection of Tumor Cells.** Bone marrow samples were obtained under local anesthesia from either one or both iliac crests of each patient through a needle aspiration and collected in heparin. Samples from the first cohort of 35 patients were enriched for tumor cells by first-line systemic chemotherapy from either one or both iliac crests of each patient through a needle aspiration and collected in heparin. Samples from the first cohort of 35 patients were enriched for tumor cells by centrifugation through a Ficoll-Hypaque density gradient (density 1.077 × g/ml; Amersham Pharmacia Biotech, Freiburg, Germany) at 900 × g for 30 min. MNCs were washed, and 10⁶ cells were centrifuged onto each glass slide at 150 × g for 5 min (15). After overnight air drying, slides were either stained immediately or stored at −20°C.

From 23 patients, bone marrow samples were taken before administration of chemotherapy at the time of surgery. At least 2 × 10⁶ MNCs were incubated with the monoclonal antibody A45-B/B3 (Micromet, Munich, Germany) at a concentration of 2 μg/ml, directed toward a common epitope of CK polypeptides, including the heterodimers CK8/18 and CK8/19 (7, 16). The slides were washed and subsequently developed by the alkaline phosphatase anti-alkaline phosphatase method as described previously (15).

**Immunocytochemical Double Labeling.** For double labeling, the murine monoclonal antibody 3B10 (Refs. 2 and 3; isotype IgG1; Micromet), directed toward an epitope of Ep-CAM, was used at 10 μg/ml. The specific reaction of the primary antibody was developed with Envision, a goat antimouse immunoglobulin conjugated to alkaline phosphatase-labeled dextran polymer (DAKO) combined with a Naphtol-AS-MX/Fast Blue reagent (Sigma). The slides were washed and subsequently incubated with the monoclonal antibody A45-B/B3 directly conjugated to the fluorochrome Cy3. Finally, slides were washed and mounted with antifade. The specificity of the antibody reaction was controlled by an equal concentration of the unrelated mouse myeloma IgG1 antibody MOPC-21 (Sigma) as an isotype control to an equal amount of MNCs as screened by the Ep-CAM-specific antibody. In a positive control, cytospins with a mixture of mouse 3T3 fibroblasts and the Ep-CAM breast carcinoma cell line BT20 were used. For each patient, between 2–4 × 10⁶ MNCs (median 4 × 10⁶ MNCs)
were screened manually by combined bright field and fluorescence microscopy. All slides were examined by two independent observers who agreed on the same result in 95% of the specimens. The final consensus decision on discrepant results required critical reevaluation by both investigators.

**Immunomagnetic Enrichment and Immunocytochemical Detection.** To specifically enrich Ep-CAM-positive cells, the subsequent 27 aspirates were treated by an immunomagnetic selection method. Bone marrow samples (2.5–5 ml) were washed once with Hanks’ buffer. The cell pellet was resuspended in 2 ml of Hanks’ buffer, and CELLlection Pan Mouse IgG immunobeads (Dynal, Oslo, Norway) conjugated with the monoclonal 3B10 antibody coupled to beads by a DNA linker were added. After incubation, labeled cells were positively selected by magnetic force. To avoid cell damage and potential interference with the subsequent detection of CK-positive cells, beads were magnetically removed from their target after DNase treatment. Enriched cells then were cytospin on slides as described above. CK-positive cells were detected by Fab fragments of monoclonal antibody A45-B/B3 that were directly conjugated to alkaline phosphatase (Micromet; 2 g/ml). The alkaline phosphatase reaction was developed by a Red New Fuchsin stain, and slides were examined by bright field microscopy.

**RESULTS**

Bone marrow samples were taken from 62 breast cancer patients without overt metastasis after treatment with various

---

**Table 1** Relation between the presence of CK positive cells (CK+) in bone marrow after adjuvant chemotherapy and patient’s characteristics

<table>
<thead>
<tr>
<th></th>
<th>No. of patients analyzed</th>
<th>No. of patients with CK+ cells enriched by Ficoll gradient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>by Ficoll gradient</td>
<td>Immunomagneticallya</td>
</tr>
<tr>
<td>Total 62</td>
<td>35 (56%)</td>
<td>27 (44%)</td>
</tr>
<tr>
<td>Age/yr (median 48.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>16 (47%)</td>
<td>18 (53%)</td>
</tr>
<tr>
<td>≥50</td>
<td>19 (68%)</td>
<td>9 (32%)</td>
</tr>
<tr>
<td>Menopausal status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Premenopausal</td>
<td>15 (52%)</td>
<td>14 (48%)</td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>20 (61%)</td>
<td>13 (39%)</td>
</tr>
<tr>
<td>Lymph node metastases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>15 (52%)</td>
<td>14 (48%)</td>
</tr>
<tr>
<td>&lt;10</td>
<td>20 (61%)</td>
<td>13 (39%)</td>
</tr>
<tr>
<td>pT status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT1</td>
<td>15 (54%)</td>
<td>13 (46%)</td>
</tr>
<tr>
<td>pT2</td>
<td>15 (52%)</td>
<td>14 (48%)</td>
</tr>
<tr>
<td>pT3/4</td>
<td>5 (100%)</td>
<td>0</td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>2 (50%)</td>
<td>2 (50%)</td>
</tr>
<tr>
<td>G2</td>
<td>19 (59%)</td>
<td>13 (41%)</td>
</tr>
<tr>
<td>G3</td>
<td>14 (54%)</td>
<td>12 (46%)</td>
</tr>
<tr>
<td>Histological type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ductal</td>
<td>26 (55%)</td>
<td>21 (45%)</td>
</tr>
<tr>
<td>Lobular</td>
<td>4 (80%)</td>
<td>1 (20%)</td>
</tr>
<tr>
<td>Other typesc</td>
<td>5 (50%)</td>
<td>5 (50%)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMFd</td>
<td>7 (41%)</td>
<td>10 (59%)</td>
</tr>
<tr>
<td>ECF</td>
<td>13 (54%)</td>
<td>11 (46%)</td>
</tr>
<tr>
<td>Other typesf</td>
<td>15 (71%)</td>
<td>6 (29%)</td>
</tr>
<tr>
<td>Radiotherapyg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>22 (55%)</td>
<td>18 (45%)</td>
</tr>
<tr>
<td>No</td>
<td>13 (59%)</td>
<td>9 (41%)</td>
</tr>
<tr>
<td>Hormone therapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes (Tamoxifen only)</td>
<td>16 (50%)</td>
<td>16 (50%)</td>
</tr>
<tr>
<td>Other typesb</td>
<td>2 (33%)</td>
<td>4 (66%)</td>
</tr>
<tr>
<td>No</td>
<td>17 (71%)</td>
<td>7 (29%)</td>
</tr>
</tbody>
</table>

a Between 2.5 and 5 ml of total bone marrow was screened.

b Between 2 and 4 times 10^6 MNCs per patient were screened by the Ficoll gradient method. Note, that using this method, no cells double positive for CK and Ep-CAM could be detected.

c Other histological types included ductal/lobular-mixed carcinoma (5 patients), desmoplastic carcinoma (2 patients), atypical medullary carcinoma (1 patient), papillary carcinoma (1 patient), and polymorph cellular carcinoma (1 patient).

d CMF, cyclophosphamide-methotrexate-5-fluorouracil. Six double courses of CMF (day 1 + 8): cyclophosphamide (500 mg/m² body square), methotrexate (40 mg/m² body square), and 5-fluorouracil (600 mg/m² body square).

e EC, epirubicin. Four courses of EC (q21): epirubicin (90 mg/m² body square) and cyclophosphamide (600 mg/m² body square).

f Other chemotherapeutic regimens consisted of different combinations of an anthracycline and a taxane, sequential combinations of EC, and CMF applied with different numbers of courses.

g Radiotherapy implicated a total dose of 50.4 Gy plus 10 Gy boost radiation.

h Other types were a combination of tamoxifen with anastrozol or a GnRH-analogon (5 patients) and raloxifene (1 patient).
forms of systemic and local adjuvant therapy. Aspirates of the bone marrow were analyzed for Ep-CAM-positive tumor cells by two different methods. As shown in Table 1, about equal numbers of patients were examined in each group. Although patients analyzed by the Ficoll density gradient method were somewhat older in comparison with the cohort examined by immunomagnetic enrichment (68 versus 32%, respectively) in the group of patients ≥50 years and presented with more lymph node involvement (63 versus 37% respectively), characteristics were similar in respect of distribution of tumor size, histological grade, type, and the form of adjuvant therapy.

In total, 10 of 35 (28.6%) bone marrow samples processed by the Ficoll gradient method were positive for cells expressing CK (Table 1). In each sample of 2–4 × 10^6 mononucleated interphase cells analyzed, only a maximum of 10 CK-positive cells could be detected, as illustrated in Fig. 1. However, double labeling of these cells showed that none of them coexpressed Ep-CAM; a representative example of positive immunostaining of CK and negative staining of Ep-CAM of the same cell is shown in Fig. 2, A, B, C, and D, whereas BT 20 breast cancer cells, used as a positive control, stain positive for both CK and Ep-CAM (Fig. 2, A and B). All CK-expressing cells presented as single isolated cells, whereas cell clusters could not be found. No significant correlation was observed between the rate of CK-positive cells and the clinicopathological characteristics of the patients, such as axillary lymph node involvement, histological tumor type, or the mode of systemic treatment of the patients (Table 1). We did not observe a significant correlation between the number of courses of chemotherapy and presence of micrometastatic cells. However, the total number of patients analyzed might be too small to exclude the existence of such a correlation.

To further enhance the sensitivity of tumor cell detection, we applied a new technique to selectively enrich for Ep-CAM^+ cells using immunomagnetic beads coupled with anti-Ep-CAM antibody 3B10. However, the analysis of this subsequent cohort of 27 patients, who did not differ significantly from the first cohort regarding the patient’s characteristics (Table 1), revealed similar results; only 2 of 27 samples analyzed (7.4%) presented with CK-positive cells coexpressing Ep-CAM.

In one of the samples, we found 36 double-positive cells. Interestingly, 21 of these cells were organized in clusters consisting of 3–7 cells (Fig. 2E). In the other sample, only 2 single isolated double-positive cells were detected.

Although not within the main focus of this study, 23 patients with available bone marrow samples that have been taken before administration of chemotherapy at the time of surgery were analyzed for the presence of CK positive cells by immunocytochemistry after enrichment by the Ficoll density gradient method. Fourteen of these 23 patients were bone marrow positive at primary surgery, and 6 of them were still positive after postoperative chemotherapy (data not shown).

To estimate the extent of Ep-CAM expression of the primary tumors, formalin-fixed and paraffin-embedded tissue sections have been stained immunohistochemically for Ep-CAM expression (Fig. 2F). We have stained tumor sections from 12 patients enrolled in the study. All of them expressed the antigen; however, the fraction of Ep-CAM-positive tumor cells varied among the different specimens between 10 and >80%.

**DISCUSSION**

Several studies performed by various research groups over the past 10 years have indicated that the presence of immunohistochemically identifiable tumor cells in bone marrow is associated with an increased risk of metastatic relapse and reduced overall survival in breast cancer (7–12, 17), as well as in many other epithelial malignancies (18–22). Therefore, defining the load of disseminated tumor cells in the bone marrow has been used as a surrogate marker to monitor the efficacy of adjuvant therapy (23–25). In this study, we were able to show that 29% of our patients analyzed by the standard Ficoll density gradient enrichment and subsequent immunohistochemical staining presented with CK^+ cells in their bone marrow after first-line adjuvant therapy. In total, the number of tumor cells identified per patient using the Ficoll density gradient method was rather low, usually between 1 and 3 per 2 × 10^6 MNCs analyzed. Nevertheless, considering a total marrow cellularity in humans of ~10^10 nucleated cells per kilogram of body weight (26), this minimal tumor load in total bone marrow may be ~2–4 × 10^5 CK^+ cells.

We did not try cell panning with anti-CK antibodies because of the known poor recovery rates. However, we tried to enrich tumor cells bound to CK antibodies using a commercially available magnetic column-based system from Miltenyi, Inc. Although this system works well with blood, the recovery rates with bone marrow samples were poor (data not shown). One reason for this discrepancy might be the fact that bone marrow samples are more complex in their composition than blood samples, leading to a blockage of the columns.

In this study, patients were only enrolled after completion of adjuvant chemotherapy because the effect of adjuvant chemotherapy on bone marrow micrometastases has already been analyzed in a previous trial by Braun et al. (25), who observed
Ep-CAM Expression on Micrometastatic Cells

Double immunostaining (CK and Ep-CAM) of disseminated carcinoma. A, cytoplasmatic CK staining (fluorescent orange dye Cy3) of 3T3 fibroblasts are unstained. B, Ep-CAM staining (Fast Blue stain) of the same cells in bright field microscopy. C, cytoplasmatic fluorescent CK staining of a single metastatic cell from a patient with breast cancer. The surrounding bone marrow cells are unstained. D, same cell in the bright field. No Ep-CAM staining can be observed. E, example of occult CK-positive cancer cells organized in a cell cluster. Cells have been enriched immunomagnetically and subsequently stained for CK (Red New Fuchsin stain). No counterstaining was performed; original magnification: ×1000. F, section from formalin-fixed and paraffin-embedded tissue. Strong Ep-CAM membrane immunostaining in the invasive (long black arrow) and intraductal (short black arrow) components of a ductal carcinoma and only weak immunostaining in normal epithelia (red arrows); original magnification: ×200.

In the present study, we therefore evaluated whether disseminated tumor cells present in the bone marrow of breast cancer patients who had received adjuvant chemotherapy express Ep-CAM. We choose this time point because adjuvant chemotherapy after the more established chemotherapeutic regimens.

Survival and partial resistance of tumor cells in bone marrow and, hence, failure of adjuvant chemotherapy may be attributable to the fact that the majority of these tumor cells appear to be nonproliferating (28), which is consistent with the extended latency period (dormancy) between their primary diagnosis and the occurrence of a subsequent metastatic relapse. In this respect, cell cycle-independent treatment strategies, such as antibody-based immunotherapy, which have been recently shown to be active in breast cancer (7, 29, 30), could be of interest. However, to select appropriate antigen targets, simple extrapolation from antigen patterns expressed by the primary tumor to those present on metastatic cells may not be sufficient (31). Instead, direct phenotyping of metastatic tumor cells is more likely to identify appropriate therapeutic target antigens.

Although Ep-CAM may be a suitable target in this regard, it has shown to be expressed in a rather heterogeneous manner in breast cancer (6, 13). Gastl et al. (6) documented only the rate of overexpression of Ep-CAM (73 of 205 primary tumors) as compared with the staining intensity of normal breast tissue. In our study on Ep-CAM expression on single micrometastatic cells, we did not take staining intensity into account, because we had no normal Ep-CAM-positive reference cell type present in the bone marrow sample. Several groups have shown that Ep-CAM expression detectable by immunohistochemistry is usually found on all breast tumors [e.g., 95% of Ep-CAM-positive tumors in the study of Packeisen et al. (4)]. We have stained tumor sections from 12 patients enrolled in our study, and our results confirm the consistent expression of Ep-CAM in breast tumors; however, the fraction of Ep-CAM-positive tumor cells varied considerably among the different specimens.

In the present study, we therefore evaluated whether disseminated tumor cells present in the bone marrow of breast cancer patients who had received adjuvant chemotherapy express Ep-CAM. We choose this time point because adjuvant immunotherapy will most likely be applied as a second-line therapy after the more established chemotherapeutic regimens. Although 29% of the samples analyzed by Ficoll density gradient expressed CK, none of them typed for Ep-CAM as well.

To further enhance the sensitivity of tumor cell detection, we applied a new technique to enrich Ep-CAM+ cells using immunomagnetic beads and were able to identify only 7.4% of the patients with cells double positive for both antigens. The
rationale for using an Ep-CAM-based immunobead selection step despite the fact that Ep-CAM was rarely detected with our double-labeling approach was based on the previous report that Ep-CAM expression might be restored on cell clusters, and we know that cell clusters can be lost by Ficoll density centrifugation (15). In fact, clusters of tumor cells could be observed only by the immunomagnetic method but not the Ficoll density method. Thus, the Ficoll density method that has been used in most studies on occult metastatic tumor cells may lead to a loss of cell aggregates (32). One can speculate that these clustered cells may represent the first step toward the development of overt metastases and that the adhesion of these clustered Ep-CAM-positive cells is mediated by Ep-CAM. However, because of the few tumor cells present in the bone marrow samples before and after chemotherapy in the 23 available patients, it might be very difficult to distinguish whether the lack of Ep-CAM expression is a true result of “single cell” biology or the chemotherapy. Unfortunately, we were not able to get access to distant metastases of these patients. Metastatic relapse in breast cancer occurs within a time frame of ≥10 years after primary diagnosis. Thus, patients might not be available anymore, and distant metastases are usually not surgically removed but treated by systemic therapies. Taken together, it is extremely difficult to get sets of autologous primary tumors and metastases from the same patients. Nevertheless, there is at least some experimental evidence that Ep-CAM expression is restored in cell clusters and maybe also in solid metastases (33).

Thus far, in the literature, there are no comparable data available about Ep-CAM expression on disseminated tumor cells in nonmetastatic breast cancer patients after chemotherapy. Our results indicate that immunotherapeutic approaches directed against Ep-CAM might be limited to the subgroup of patients with residual Ep-CAM-positive tumor cells. Because of both the small sample size and fact that only a few tumor cells per sample could be detected, this subgroup might be underestimated to some extent. Nevertheless, additional therapeutic targets need to be identified to completely eradicate all residual cancer cells (13, 34).

REFERENCES

Rare Expression of Epithelial Cell Adhesion Molecule on Residual Micrometastatic Breast Cancer Cells after Adjuvant Chemotherapy

Holger Thurm, Sebastian Ebel, Christina Kentenich, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/9/7/2598

Cited articles
This article cites 27 articles, 9 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/9/7/2598.full#ref-list-1

Citing articles
This article has been cited by 6 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/9/7/2598.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://clincancerres.aacrjournals.org/content/9/7/2598.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.