Monoclonal Antibody Therapy with Edrecolomab in Breast Cancer Patients: Monitoring of Elimination of Disseminated Cytokeratin-positive Tumor Cells in Bone Marrow

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ABSTRACT

Despite current advances in antibody-based immunotherapy of breast and colorectal cancer, we have recently shown that the actual target cells (e.g., tumor cells disseminated to bone marrow) may express a heterogeneous pattern of the potential target antigens. Tumor antigen heterogeneity may therefore represent an important limitation of the efficacy of monospecific antibody therapy. To measure the efficacy of such a monospecific approach, we analyzed the elimination of tumor cells coexpressing the epithelial cell adhesion molecule (EpCAM) under therapy with murine monoclonal antibody 17-1A (Edrecolomab) directed against EpCAM. In bone marrow aspirates from 10 breast cancer patients with metastatic tumors (n = 8) and locoregional recurrence (n = 2), tumor cells were identified with the antibody A45-B/B3 directed against the epithelial differentiation marker cytokeratin (CK) and simultaneously typed for EpCAM expression using the antibody 17-1A. Patients were treated with a single dose of 500 μg of Edrecolomab and monitored by bone marrow analyses before and at days 5–7 after antibody treatment. In all 10 patients, we assessed a marked reduction in the mean numbers of both CK+ cells (73 versus 15; P = 0.003, t test) and EpCAM+/CK+ cells (17 versus 1; P = 0.003, t test) per 10⁶ bone marrow cells. Complete elimination of EpCAM+ cells was possible in four patients. We conclude that Edrecolomab can be used in breast cancer patients to target isolated EpCAM+/CK+ cancer cells. Using CK-based immunoassays, we reliably detected residual tumor cells in bone marrow and typed EpCAM expression. This allowed us to monitor the cytotoxic elimination of such cells after Edrecolomab application. Selection of EpCAM+/CK+ tumor clones showed that further antibodies directed against tumor-associated antigens are warranted to improve the efficacy of monospecific approaches.

INTRODUCTION

A reliable indication of the efficacy of adjuvant therapy requires trials with large numbers of patients observed for several years (1), especially in breast cancer, because residual tumor cells may exert their influence on survival at 10 years or later (2). Because adjuvant treatment usually is delivered to patients with clinically occult micrometastatic disease after the successful resection of the primary tumor, the efficacy of therapy can be only assessed retrospectively from the rate of disease-free survival. Consequently, progress in this form of therapy is extremely slow and cumbersome, and therapy is difficult to tailor to the special needs of an individual patient. The importance of a surrogate marker assay that would permit the immediate assessment of therapy-induced cytotoxic effects on residual cancer cells is therefore obvious.

The immunocytotoxic detection of hematogenously disseminated tumor cells may represent such a surrogate marker assay because numerous studies have demonstrated the prognostic impact of such early tumor cell dissemination in breast cancer patients [e.g., reviewed in Ref. (3)]. In recent studies, we and others have demonstrated the validity of the immunoassay with antibodies directed against CK as specific marker antigen of extrinsic epithelial cells in the background of mesenchymal bone marrow cells (4–8). In this study, we have therefore applied the monoclonal antibody A45-B/B3 directed against the heterodimers CK8/18 and CK8/19 as well as a common epitope of several CK polypeptides (4, 9).

Cytotoxic chemotherapy regimens currently applied for advanced breast cancer might fail to eliminate dormant, nonproliferating tumor cells (10), which may explain metastatic relapse after chemotherapy, and even after high-dose chemotherapy (11, 12). In this view, cytotoxic antibodies represent a promising therapeutic option for the specific treatment of minimal residual disease (13). Nevertheless, epithelial cancer cells are known for their genetic instability, which generates a high degree of heterogeneity of the different tumor cell clones found in an individual tumor. Using double-labeling techniques, we previously have shown that this tumor antigen heterogeneity can
Monitoring of Edrecolomab Cytotoxicity in Breast Cancer

The recognition of therapy-resistant tumor cell clones is crucial for the assessment of immediate efficacy in immunotherapy trials and for the development of future immunotherapies. Despite the implementation of such approaches, a heterogeneous antigen-profile of disseminated tumor cell clones might limit the efficacy of current treatment strategies.

In the present study, we investigated the effect of a monoclonal antibody directed against EpCAM, which is known to be expressed on tumor cells of various origins, including breast cancer. Our data suggest that tumor cells in bone marrow can be reliably detected and typed for antigen expression, allowing the monitoring of cytotoxic elimination of such cells after Edrecolomab application. The consistency of the findings advocates the implementation of immunocytochemical monitoring into the design of future immunotherapy trials for immediate toxicity estimation and recognition of the selection of therapy-resistant tumor cell clones.

PATIENTS AND METHODS

Patients. Bone marrow aspirates from 21 patients with breast cancer were subjected to bone marrow aspiration from both upper iliac crests under local anesthesia with 1% mepivacain-HCl. Only patients who presented with micrometastases, as screen for the presence of breast cancer micrometastases, the antibody A45-B/B3 (Micromet) directed against EpCAM, and the antibody 17-1A (Edrecolomab) directed against EpCAM, were included in the procedures of immunocytochemical monitoring and into the design of future immunotherapy trials for immediate toxicity estimation and recognition of the selection of therapy-resistant tumor cell clones.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Site of recurrence</th>
<th>Prior to treatment</th>
<th>After treatment</th>
<th>Related toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bone, lung</td>
<td>97(^a)</td>
<td>2(^a)</td>
<td>Diarrhea (grade I)</td>
</tr>
<tr>
<td>2</td>
<td>Bone, liver, lung</td>
<td>227</td>
<td>26</td>
<td>Urticaria and mild bronchospasm (grade III)</td>
</tr>
<tr>
<td>3</td>
<td>Bone</td>
<td>67</td>
<td>11</td>
<td>Diarrhea (grade I)</td>
</tr>
<tr>
<td>4</td>
<td>Lung</td>
<td>17</td>
<td>5</td>
<td>Diarrhea (grade I)</td>
</tr>
<tr>
<td>5</td>
<td>Lung</td>
<td>115</td>
<td>20</td>
<td>Diarrhea (grade I)</td>
</tr>
<tr>
<td>6</td>
<td>Locoregional</td>
<td>10</td>
<td>2</td>
<td>None</td>
</tr>
<tr>
<td>7</td>
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<td>5</td>
<td>0</td>
<td>Diarrhea (grade I)</td>
</tr>
<tr>
<td>8</td>
<td>Bone</td>
<td>35</td>
<td>6</td>
<td>Diarrhea (grade I)</td>
</tr>
<tr>
<td>9</td>
<td>Bone, liver</td>
<td>150</td>
<td>37</td>
<td>None</td>
</tr>
<tr>
<td>10</td>
<td>Lung</td>
<td>10</td>
<td>3</td>
<td>None</td>
</tr>
</tbody>
</table>

\(^a\) Single APAAP immunostaining: values given as number of CK\(^+\) cells per 2 \times 10^6 mononuclear cells.

\(^b\) Immunogold-alkaline phosphatase double-labeling: values are given as number of EpCAM\(^-\)CK\(^+\) (double-positive) cells per total number of CK\(^+\) cells (%).

Edrecolomab (500 mg; Glaxo-Wellcome) was given as a single dose intravenously. The antibody infusion was prepared in sterile glass bottles containing 250 ml of 0.9% NaCl and infused over a time period of 2 h with continuous monitoring of vital signs. During the study period, patients received no further antitumoral therapy. Written informed consent was obtained from all patients. The study has been approved by the local board of the Ethics Committee.

Tissue Preparation. The volumes of all bone marrow aspirates yielded a mean of 1.75 \times 10^7 mononuclear cells. After centrifugation through a Ficoll-Hypaque density gradient (density, 1.077 g/ml; Pharmacia) at 900 \times g for 30 min, interface cells were washed, and 10^6 cells were centrifuged onto each glass slide at 150 \times g for 5 min (19). After overnight air-drying, slides were either stained immediately or stored at −80°C.

Immunocytochemical Bone Marrow Screening. To screen for the presence of breast cancer micrometastases, the antibody A45-B/B3 (Micromet) directed against EpCAM was used at a concentration of 0.9% NaCl and infused over a time period of 2 h with continuous monitoring of vital signs. During the study period, patients received no further antitumoral therapy. Written informed consent was obtained from all patients. The study has been approved by the local board of the Ethics Committee.

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Immunocytochemical Double Labeling. The double-labeling technique consisted of simultaneous incubation with alkaline phosphatase-conjugated anti-Ck A45-B/B3 Fab fragments (Micromet) and anti-EpCAM immunoglobulin Edrecolomab (Glaxo Wellcome), as described previously (19). Prior to any incubation steps, slides were permeabilized with 0.1% Triton X-100 (Boehringer) for 10 min and fixed with 1.0% paraformaldehyde (Merck) for 20 min at 4°C. All washing steps were carried out in PBS buffer (pH 7.4) unless otherwise noted. After an initial blocking step with 0.1% linearized BSA (Aurion) for 10 min, both antibody conjugates were co-incubated for 45 min. In a second incubation step, Fc terminus-
specific goat antiamouse immunoglobulins conjugated with colloidal gold particles (Amersham) were added to specifically label murine Edrecolomab. Subsequently, slides were exposed to 2.0% glutaraldehyde with washing steps prior to and after fixation that were performed in H2O to eliminate chloride ions known to interfere with the subsequent silver precipitation. The silver development was carried out with a Silver Enhancement Kit (Amersham) according to the manufacturer’s recommendations. Silver development was closely monitored under the microscope and terminated as soon as brown to black silver precipitates became visible, usually after 15–23 min. Appropriate control slides with the BT-20 breast cancer cell line were included in all stainings. Finally, slides were developed with new fuchsin solution for 20 min, as indicated above.

RESULTS

The bone marrow aspirates in the present study were obtained from breast cancer patients with an advanced disease stage. As shown in Table 1, 8 of 10 patients had distant metastases manifested at least one site, whereas the remaining 2 patients displayed locoregionally recurrent disease. All patients had a stable disease stage without clinical signs of disease progression at the time of inclusion into the study. In contrast to early stage patients, this selection increased the likelihood of the presence of hematogenously disseminated tumor cells in relevant secondary sites of metastasis, such as bone marrow. Overall, 15 of 21 patients (71%) screened for this study presented with a CK+ finding in bone marrow, which is consistent with our previous findings on screening advanced breast cancer patients (21). In these 15 patients, the amount of CK-positivity ranged from 1 to 224 CK+ cells per 106 bone marrow cells. For a meaningful interpretation of therapeutic antibody activity, we decided to exclude the five patients with <5 CK+ cells per 106 bone marrow cells.

Thus, at the time of initiation of antibody treatment, a mean of 73 (range, 5–224) CK+ tumor cells per 106 bone marrow cells were detected in the patients of the study population (Table 1). Under the assumption of a homogeneous distribution, the mean total tumor load present in the bone marrow of these 10 patients was 5.8 × 107 (4 × 106 to 1.8 × 108) cells (22). After the application of 500 mg of Edrecolomab, a marked reduction of this tumor load was found (P = 0.003, t test for paired samples) in all follow-up bone marrow aspirates performed between days 5 and 7, as shown in Fig. 1A. Thus, the total tumor load in bone marrow was reduced to a mean of 8.8 × 106 (0 to 3.0 × 107) cells.

To demonstrate that the described reduction was due to antibody cytotoxicity, we used double-labeling to type CK+ cells for the coexpression of the target antigen EpCAM. This analysis was performed on another set of slides of the same bone marrow aspirate, which explains the different numbers of tumor cells compared with the single-labeling results described above (Table 1). In double-labeling, CK+ breast cancer cells in bone marrow were identified by Fab fragments of the antibody A45-B/B3 conjugated directly with alkaline phosphatase, which identified cancer cells with sensitivity equal to that of the standard APAAP procedure in single labeling (Spearman’s correlation coefficient r = 0.967; P = 0.0037). The mean percentage of EpCAM+/CK+ cells per total number of CK+ cells was 68% (48–100%), which is consistent with our previous findings (14). Thus, the actual targets of antibody therapy were reliably detected by the applied double-labeling technique. Similar to the reduction of CK+ tumor cells, the number of EpCAM+/CK+ cells detected after administration of 500 mg of Edrecolomab was significantly lower than before antibody treatment (P = 0.003, t test for paired samples), as shown in Fig. 1B. Fig. 2 displays examples of EpCAM+/CK+ tumor cells before Edrecolomab infusion and EpCAM+/CK+ tumor cells after Edrecolomab infusion.

Because the study population comprised patients in ad-
DISCUSSION

The primary goal of this study was to determine whether the known antibody-dependent cytotoxicity of Edrecolomab (18) could be monitored in advanced breast cancer patients by repeating immunocytochemical bone marrow aspiration before and after antibody infusion. We were able to demonstrate that typing for EpCAM expression of micrometastatic breast cancer cells defined these cells as suitable targets of Edrecolomab, which was originally thought to be a cytotoxic agent for colorectal cancer cells only. Because extrapolation from antigen patterns expressed by the primary tumor has been shown to be unreliable (23), phenotyping of bone marrow micrometastases by double-labeling techniques thus may help enlarge the therapeutic spectrum of monoclonal antibody therapy. On the basis of our present data, we believe that antigen expression by micrometastatic cells may be a better predictor of response to antibody-based therapy than expression by the related primary tumor. Our pilot study provides data that may form the basis for conducting larger trials to relate a certain reduction of micrometastatic tumor load to defined clinical end points, such as disease-free and overall survival.

The reliability of CK-based immunoassays, both single- and double-labeling, to detect micrometastatic cells in bone marrow has been demonstrated in numerous studies that included carcinoma patients as well as patients with no evidence for malignant disease (4, 5, 10, 14, 24). Further justification for the use of CK as a marker of epithelial tumor cells in bone marrow derived from studies showing that CK⁺ cells carry the typical genomic hallmarks of malignant cells (25). Recently, we also demonstrated that the validated detection of disseminated tumor cells as relevant precursors of metastatic relapse is prognostically important (3).

The reproducibility of this assay is determined by an unavoidable sampling error that appears to be an important issue of the presented approach. A caveat therefore needs to be added because false-negative bone marrow results cannot be completely excluded. Interpretation of the appearance or disappearance of relatively few CK⁺ cells as success or failure of the applied therapy—if done carefully—appears to be possible, as shown in previous monitoring studies with micrometastatic tumor cells as surrogate markers of efficacy (26, 27). On the basis of these findings, we have improved the sensitivity of our CK assay for the present study by increasing the number of mononucleated cells analyzed before and after therapy from $4 \times 10^5$ to $1 \times 10^6$ and have used the more sensitive antibody A45-B/B3 (4). In addition, we typed CK⁺ cells for EpCAM, i.e., target antigen coexpression, to generate a second and direct marker for the therapeutic activity of Edrecolomab. For reliable quantification, we excluded patients with a tumor load $<5$ CK⁺ cells per $10^6$ bone marrow cells, the lowest number that appeared to be reproducibly detectable (14). Although new developments in the enrichment of tumor cells using immunomagnetic beads are promising, the reproducibility of this new technology is still under investigation (28). Thus, we assume that our precautions gave a reliable selection of patients for monitoring effects of antibody cytotoxicity rather than assessing only random fluctuations of cell counts.

To minimize the interference by cell death over time, which might have been independent of the antibody’s cytotoxic action, we chose to assess the reduction of EpCAM⁺ cancer cells after a relatively narrow interval of 5–7 days, and we excluded patients receiving ongoing antitumoral treatment from the study. Although the total number of patients analyzed was small and the measured tumor cell reduction in an individual patient might be questionable, the consistent reduction of CK⁺ cells in all patients (Fig. 1) strongly supports the conclusion that EpCAM⁺ micrometastatic breast cancer cells are susceptible to i.v. treatment with Edrecolomab. Therefore, it is likely that the observed reduction or eradication of EpCAM⁺/CK⁺ cells is an effect of the infused antibody. Regarding the six patients who
had EpCAM+/CK+ cells after Edrecolomab infusion, it may be speculated that elimination of tumor cells was incomplete within this short period because the half-life of Edrecolomab in the serum is ~10 days (15). The presumably underlying phenomenon of in vivo antibody-labeled tumor cells in bone marrow was demonstrated in a previous study by direct visualization of tumor cells carrying 125I-conjugated in vivo labeled antibodies after i.v. application (6). It is thus conceivable that EpCAM+ cancer cells detectable within 5–7 days after treatment may be in vivo Edrecolomab-labeled cells that have not yet been eliminated by the host’s immune system. Because this remains speculative in the setting of our pilot study, patients of our study population received adequate standard cytotoxic or immunotherapeutical treatment immediately after the second bone marrow aspiration because we were aware of the potentially insufficient treatment of metastatic breast cancer by this single-antibody infusion alone. Thus, follow-up aspirations to assess the long-term effects of antibody infusion on tumor cells in bone marrow were not possible for ethical reasons.

Among the first studies that displayed the biological effect of an unconjugated antibody against clinically occult micrometastatic tumor cells was an adjuvant trial on patients with Dukes’ C colorectal cancer receiving Edrecolomab that revealed a significant reduction in both mortality and morbidity (18). Because chemotherapy, however, plays an irrefutable role in breast cancer treatment, combinations of antibody therapy and chemotherapy might be complementary for the elimination of dormant micrometastases. This assumption has been sustained by recent trials showing improved outcome of metastatic breast cancer patients who received trastuzumab (anti-HER2 antibody) as an additive to first-line chemotherapy (29). The moderate rate and duration of measurable response in such advanced breast cancer patients, however, can be explained by the significant tumor burden as well as the fact that solid metastases have established physiological barriers (e.g., high intratumoral oncotic pressure) that prevent access of macromolecules (e.g., antibodies) from the circulation into the metastatic lesion (30). Thus, it is clear that a major consideration for the successful application of antibody therapy is the choice of the appropriate disease stage (e.g., micrometastatic tumor cells as in our study) in which the tumor cells are accessible for i.v. administered immunoglobulins.

To date, no direct data have been published that correlate a certain reduction of micrometastatic disease to defined clinical endpoints, such as disease-free or overall survival. However, we have conducted a study demonstrating that the persistent identification of bone marrow micrometastases after adjuvant chemotherapy was associated with a significantly reduced overall survival. Using micrometastatic tumor cells in bone marrow as a surrogate model for therapeutic efficacy of neoadjuvant androgen deprivation, a recent immunocytochemical monitoring study on stage C prostate cancer patients found an association between disease-free survival and reduction of bone marrow micrometastases (26). For antibody therapy, the described bone marrow assay can be used to directly assess this susceptibility to treatment (e.g., expression of target antigens) prior to therapy. Immediately after therapy, important information on the degree of selection of therapy-resistant tumor cell clones would also be available. In future trials of larger scale than this pilot study, it will be interesting to see whether the presented bone marrow assay fulfills the expectations of a surrogate marker model for immediate monitoring of the efficacy of anticancer therapy against micrometastatic disease that would relieve the burden of using the 5-year survival count as the sole assessment of therapeutic efficacy.

REFERENCES

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