Frequent Down-Regulation of Major Histocompatibility Class I Antigen Expression on Individual Micrometastatic Carcinoma Cells


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Abstract

An astonishingly high frequency of micrometastatic cells have been found in bone marrow aspirates of patients with colon carcinomas (G. Schlimok et al., J. Clin. Oncol., 8:831–837, 1990), although these tumors very rarely metastasize to the skeleton. This observation has raised questions about the malignant potential of such cells. In a first attempt to characterize this potential, we have assessed the expression of major histocompatibility complex (MHC) class I antigens on bone marrow micrometastases, inasmuch as down-regulation of these molecules is a potential mechanism to escape from MHC class I–restricted lysis by cytotoxic T-cells. The two groups of cancer patients compared were those with tumors known to rarely (stomach and colon cancer) or frequently (breast cancer) manifest skeleton metastases. Bone marrow aspirates taken from these patients were probed for individual disseminated tumor cells using the immunoperoxidase technique with monoclonal antibody CK2 to the epithelial differentiation antigen cytokeratin 18 (CK-18), as described previously (G. Schlimok et al., Proc. Natl. Acad. Sci. USA, 84: 8672–8676, 1987). Specimens containing CK18-positive cells were labeled with monoclonal antibody W6/32 directed to a framework (or nonpolymorphic) antigenic determinant of MHC class I heavy chains associated with γ–microglobulin. W6/32-positive CK-18-positive cells could be detected in 25 of 54 patients (46.2%) with significantly higher incidences in 26 breast cancer patients (61.9%) as compared to 28 patients with carcinomas of the stomach and colon (27.3% and 29.4%). Independent from the origin of the primary carcinoma, the incidence of W6/32-negative CK18-positive cells was positively correlated to both the differentiation grade of the primary tumor (P < 0.05) and appeared to be linked to the occurrence of regional lymph node metastases (statistically not significant) determined by conventional histological examination. The present results demonstrate for the first time that down-regulation of MHC expression on individual micrometastatic cells correlates to the differential pattern of metastasis obtained by comparing breast and gastrointestinal carcinomas. This finding together with the suggestive link to clinical risk factors supports the significance of reduced MHC class I expression on the survival of residual metastatic cells which is a major determinant of prognosis for patients with solid tumors.

Introduction

Occult metastases present in many patients with small primary carcinomas pose a major risk for a late relapse in the most common forms of cancer. This “dark stage” of minute metastases can now be elucidated by identifying disseminated epithelial cells in mesenchymal organs such as bone marrow with monoclonal antibodies directed to epithelial differentiation antigens (for review, see Ref. 1). As work of several groups including ours has demonstrated, such disseminated cells occur with an astounding frequency in the bone marrow of patients with a locally restricted primary cancer (2, 3). In fact, the high incidence of these cells in patients with primary colon carcinomas (3) known to metastasize notoriously seldom to the skeletal suggests that the majority of micrometastatic cells will not grow into manifest metastases during the remaining life span of these patients.

It was therefore obvious to search for additional markers on those cells by which their malignant potential could be assessed. Among the various markers defining structures on primary tumors associated with a high risk of relapse, down-regulation of MHC class I expression has gained particular attention as a possible mechanism by which tumor cells can escape from lysis by cytotoxic T-lymphocytes (for review, see Refs. 4 and 5). Especially individual micrometastatic cells present in the bone marrow should be preferential targets, because of the high effector:target cell ratio.

This work demonstrates for the first time that down-regulation of MHC class I expression can be detected on individual micrometastatic tumor cells present in bone marrow aspirates of patients with various adenocarcinomas. Moreover, the data proffer circumstantial evidence that down-regulation of this extent might be relevant for the survival and subsequent outgrowth of such cells, suggesting a benefit of adjuvant therapy with MHC class I–inducing cytokines such as γ-interferon and tumor necrosis factor α.

Materials and Methods

Patients and Bone Marrow Aspirates. After informed consent, 54 patients with primary adenocarcinomas of the breast, stomach, or colon were investigated; each tumor was confirmed, typed, and graded (grade 2, moderately differentiated; grade 3, poorly differentiated). After an extensive diagnostic program, the patients were staged according to the tumor-nodes-metastasis classification.

Bone marrow samples were aspirated from the posterior iliac crest at the time of primary surgery. A mean volume of 5 ml of bone marrow was obtained per aspiration, yielding an average of 6 × 10⁶ nucleated cells. After density centrifugation through Ficoll-Hypaque, interface cells were cytocentrifuged on glass slides (8 × 10⁴ cells/slide). Following overnight air drying and subsequent fixation with acetone for 10 min, slides were either stained immediately or stored at -80°C. For each specimen, the cells were cytocentrifuged on glass slides (8 × 10⁴ cells/slide). Following overnight air drying and subsequent fixation with acetone for 10 min, slides were either stained immediately or stored at −80°C. For each patient, five slides were examined, while two additional slides served as immunoglobulin isotype controls. All slides were examined by two independent observers in a double blinded fashion.

Immunocytochemistry. For double labeling, two immunocytochemical techniques were combined, as described previously (6). Briefly, endogenous peroxidase activity of marrow cells was blocked by preincubation with 0.25% hydrogen peroxide (Sigma, Deisenhofen, Germany). In the first step, supernatant containing mAb W6/32 (IgG2a) directed to a framework (or nonpolymorphic) antigenic determinant of MHC class I heavy chains associated with β₂-microglobulin (7) (kindly provided by Dr. D. Schendel, Institute for Immunology, Munich, Germany) was used undiluted due to our previous testing on appropriate specimens. The antibody reaction was developed with an immunoperoxidase technique using peroxidase-conjugated rabbit anti-mouse immunoglobulin G (IgG2a), followed by goat anti-rabbit IgG (IgG2b) (Dako, Nordhavn, Denmark) directed to a second framework (or nonpolymorphic) antigenic determinant.

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2 To whom requests for reprints should be addressed, at Institut für Immunologie der LMU München, Goethestrasse 31, W-8000 Munich 2, Germany.

3 The abbreviations used are: MHC, major histocompatibility complex; mAb, monoclonal antibody; CK-18, cytokeratin 18; CK-18*, CK-18-positive.
munoglobulins purchased from Dakopatts, Hamburg, Germany. Each incubation was followed by washing in phosphate-buffered saline (pH 7.4).

Before proceeding to the next step, slides were blocked by 20 min incubation with mouse serum. Subsequently, biotinylated mAb CK2 (IgG1) (kindly provided by Dr. Bodenmüller, Boehringer Mannheim, Tutzing, Germany) was used as primary antibody for immunocytochemical detection of epithelial cells in bone marrow (1–3) at a concentration of 5 µg/ml. CK2 recognizes the intracellular cytokeratin component No. 18 present in all cells of simple epithelial including tumor cells derived thereof (8, 9). The antibody reaction was developed with streptavidin-conjugated alkaline phosphatase complexes obtained from Di-anova, Hamburg, Germany. Finally, peroxidase and alkaline phosphatase activity was revealed with aminoethylcarbazole and fast blue BB (Sigma, Deisenhofen, Germany), respectively.

Appropriate dilutions of the respective IgG subtypes derived from mouse myeloma cells served as isotype controls: UPC 10 (IgG2α) and MOPC 21 (IgG1), both purchased from Sigma, Deisenhofen, Germany. In addition, slides with HT-29 colon carcinoma cells, obtained from the American Type Culture Collection, Bethesda, MD (ATCC HTB38), were used as controls for positive staining.

RESULTS

Immunocytological Assessment of MHC Class I Expression on Micrometastatic Cells. Using our double labeling procedure, epithelial cells were identified by the blue cytoplasmatic staining of their cytokeratin 18 (Fig. 1). In contrast, the surrounding nonepithelial cells lacked cytokeratin but their cell membrane stained brown, which indicated expression of MHC class I molecules. Depending on the absence or presence of MHC class I molecules, CK-18* cells appeared as single labeled (blue) or double labeled (blue/brown) cells, respectively.

In analogy to the presentation of data obtained by flow cytometric analysis, Fig. 2 presents the distribution curves of frequencies of CK-18* cells in individual patients. The number of events per channel is replaced by the number of patients exhibiting a certain frequency of CK-18* cells in bone marrow, as indicated on the abscissa. The total number of patients with CK-18* cells expressing MHC class I molecules (■) was considerably higher than that of patients with CK-18* cells lacking MHC class I molecules (□) (29 patients or 53.7% of all cases versus 18 patients or 33.3% of all cases, respectively). However, the shapes of the two distribution curves were similar with the majority of patients exhibiting one to three CK-18* cells per sample. Seven patients (13.0%) displayed a heterogeneous pattern of class I expression; i.e., both class I-positive and class I-negative CK-18* cells were detected in the same sample (data not shown). Recently, we have demonstrated the absence of CK-18* cells in the marrow of 102 non-tumor-bearing patients, indicating that CK-18* cells in marrow of cancer patients represent disseminated epithelial tumor cells (3).

Correlation to the Origin, Differentiation Grade, and Stage of the Primary Tumor. Table 1 shows the frequency of class I expression on individual CK-18* cells in marrow of patients with adenocarcinomas of various origin. CK-18* cells derived from bone marrow of patients with carcinomas of the breast expressed class I molecules in 34.6%, while the corresponding incidences in patients with colon or stomach cancer were about twice as high (70.6 and 72.7%). The differences between breast cancer and carcinomas of the colon (P < 0.05) and stomach (P < 0.02) were statistically significant (χ² test).

Table 2 shows the expression of class I molecules in correlation to the histological grade of the primary tumor. For 10 patients, a grading was not performed, because of inoperability of the tumor. However, the overall rates of presence or absence of class I expression in the remaining 44 patients were almost identical to the corresponding rates observed in the entire group of patients (Table 1). In patients with "moderately differentiated" (grade 2) primary tumors, class I expression was almost twice as frequent as in the group of patients with "poorly differentiated" (grade 3) primary tumors (69.6 versus 38.1%). This difference was statistically significant (P < 0.05), when negative and heterogeneous expressors were combined and compared to positive expressors.

As demonstrated in Table 3, class I expression on CK-18* cells in marrow was not significantly influenced by the tumor
The differences between breast cancer patients and patients with carcinomas of the colon were detected with similar incidences (55.6 or 34.6, respectively) and were statistically significant (χ² test) comparing the ratios of positive and negative expressors.

**DISCUSSION**

In view of its role as restriction element for cytotoxic T-lymphocytes (4, 5), MHC class I antigen expression on individual disseminated tumor cells is an issue of considerable interest, because these cells may serve as particularly vulnerable targets. However, in contrast to the wealth of information on MHC class I expression on primary tumors (for review, see Ref. 4), almost nothing is known about such expression on micrometastases. This despite the fact that residual occult micrometastases pose a major risk for a late relapse in the most common forms of cancer. In the present study, we therefore applied an immunocytochemical double labeling technique to evaluate MHC class I expression on such cells present in bone marrow aspirates of patients with various adenocarcinomas.

mAbs to CK-18 have been proved to be sensitive and specific probes for the detection of individual micrometastatic tumor cells in bone marrow of patients with mammary and colorectal adenocarcinomas (for review, see Ref. 1). The observation that the presence of CK-18 cells was associated with both the occurrence of manifest metastases (2, 3) and a reduced relapse-free survival rate (3) supports the biological and clinical relevance of individual disseminated CK-18 cells.

Although previous investigations suggested that CK-18 might be expressed in normal bone marrow cells (10–12), we failed to detect cytokeratin-positive cells in bone marrow aspirates from 102 patients without clinical evidence for a malignant epithelial disease (3). However, such cells were recently detected in two patients displaying a tubulovillous adenoma of the colon. In one of these patients, a colon carcinoma was diagnosed 10 months later at a different site of the colon, suggesting that tumor cell dissemination might have already occurred before the primary tumor could be detected. Thus, the incidence of "false positive" results, if existent, seems to be very low.

The techniques applied for cell labeling are two well-known enzymatic methods (immune peroxidase and alkaline phosphatase) recently used in combination for immunocytochemical characterization of cell surface antigens (6). The choice of the antibody W6/32 was motivated by its broad specificity against class I molecules encoded by all of the classical HLA loci (7). Therefore, the staining by mAb W6/32 of virtually all nucleated bone marrow cells could be used as an internal "positive" control (Fig. 1). Since the MHC analysis was based on very few tumor cells per sample, it was also important to demonstrate that the differences obtained in terms of MHC class I expression did not simply reflect differences in the number of CK-18+ cells examined (Fig. 2).

The number of patients investigated thus far is too small to provide conclusive data concerning the clinical significance of our finding. However, the high incidence of MHC class I down-regulation on micrometastatic breast cancer cells (Table 1) is consistent with the frequent manifestation of bone metastases in those patients. In contrast, micrometastatic cells from adenocarcinomas of the gastrointestinal tract known seldom to form solid metastases in the skeleton exhibited less frequently reduced MHC class I expression (Table 1). Furthermore, the overall incidences obtained in our investigation were generally higher than those reported for primary breast or gastrointestinal carcinomas, ranging from 37 to 47% (13–18) or 3.3 to 13.0% (19–22), respectively. Taken together, these findings support the view that reduced MHC class I expression might indeed represent a selective advantage for the survival and subsequent outgrowth of disseminated carcinoma cells in the bone marrow.

Nevertheless, the correlation between MHC class I expression and the clinical risk factors revealed some apparently controversial data. On the one hand, down-regulation of MHC class I expression was associated with poor differentiation of the primary tumor (Table 2, P < 0.05) and appeared to be linked to regional lymph node involvement (Table 3, statistically not significant). On the other hand, we failed to detect a link between MHC class I expression on micrometastatic cells and clinical diagnosis of manifest distant metastases in the individual patient (Table 3). The reason for this

### Table 1 MHC class I expression on CK-18-positive cells in bone marrow and origin of the primary tumor

<table>
<thead>
<tr>
<th>Origin of primary tumor</th>
<th>No. of patients/group</th>
<th>No. of patients with MHC class I+ CK-18+ cells in marrow</th>
<th>Positive</th>
<th>Positive/negative</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>26</td>
<td>9 (34.6)</td>
<td>4 (15.4)</td>
<td>13 (50.0)</td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td>17</td>
<td>12 (70.6)</td>
<td>1 (5.9)</td>
<td>4 (23.5)</td>
<td></td>
</tr>
<tr>
<td>Stomach</td>
<td>11</td>
<td>8 (72.7)</td>
<td>2 (18.2)</td>
<td>1 (9.1)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>54</td>
<td>29 (53.7)</td>
<td>7 (13.0)</td>
<td>18 (33.3)</td>
<td></td>
</tr>
</tbody>
</table>

* As defined with mAbs CK2 and W6/32 in double labeling.

### Table 2 MHC class I expression on CK-18 positive cells in bone marrow and differentiation grade of primary tumor

<table>
<thead>
<tr>
<th>Differentiation grade of primary tumor</th>
<th>No. of patients/group</th>
<th>No. of patients with MHC class I+ CK-18+ cells in marrow</th>
<th>Positive</th>
<th>Positive/negative</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 2</td>
<td>23</td>
<td>16 (69.6)</td>
<td>2 (8.7)</td>
<td>5 (21.7)</td>
<td></td>
</tr>
<tr>
<td>Grade 3</td>
<td>21</td>
<td>8 (38.1)</td>
<td>5 (23.8)</td>
<td>8 (38.1)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>44</td>
<td>24 (54.5)</td>
<td>7 (15.9)</td>
<td>13 (29.5)</td>
<td></td>
</tr>
</tbody>
</table>

* As defined with mAbs CK2 and W6/32 in double labeling.

### Table 3 MHC class I expression on CK-18 positive cells in bone marrow and tumor stage

<table>
<thead>
<tr>
<th>Tumor stage</th>
<th>No. of patients/group</th>
<th>No. of patients with MHC class I+ CK-18+ cells in marrow</th>
<th>Positive</th>
<th>Positive/negative</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage M0</td>
<td>36</td>
<td>20 (55.6)</td>
<td>4 (11.1)</td>
<td>12 (33.3)</td>
<td></td>
</tr>
<tr>
<td>Lymph node negative</td>
<td>19</td>
<td>11 (57.9)</td>
<td>3 (15.8)</td>
<td>5 (26.3)</td>
<td></td>
</tr>
<tr>
<td>Lymph node positive</td>
<td>17</td>
<td>9 (52.9)</td>
<td>1 (5.9)</td>
<td>7 (41.2)</td>
<td></td>
</tr>
<tr>
<td>Stage M1</td>
<td>18</td>
<td>9 (50.0)</td>
<td>3 (16.7)</td>
<td>6 (33.3)</td>
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<tr>
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discrepancy remains speculative. One possible explanation would be that down-regulation of MHC class I expression may be important only as long as the metastatic tumor load remains small, because T-cell-mediated lysis can be most effective if the effector:target cell ratio is high. With the occurrence of clinically detectable metastases, the situation becomes more complex which could lead to the disappearance of a preexisting relevance of MHC class I expression on metastatic cells.

Although the clinical relevance of the present findings needs to be confirmed, the prepared approach might be useful to identify carcinoma patients with minimal residual cancer who are at higher risk to develop metastatic disease and could profit from treatment with MHC class I-inducing agents, such as γ-interferon and tumor necrosis factor α (23, 24).

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