Tumor Angiogenesis and Micrometastasis in Bone Marrow of Patients with Early Gastric Cancer

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ABSTRACT
In a subset of patients with early gastric cancer, there were recurrences of the disease after a curative resection had been done. Direct evidence of tumor seeding in distant organs at the time of surgery for gastric cancer is not available. An immunocytochemical assay for epithelial cytokeratin protein may fill this gap because it is a feature of epithelial cells that would not normally be present in bone marrow. From 1994–1997, the bone marrow of 45 patients with early gastric cancer was examined for tumor cells, using immunocytochemical techniques and an antibody reacting with cytokeratin, a component of the intracytoplasmatic network of intermediate filaments. Intratumoral microvessels were stained with anti-CD31 monoclonal antibody. Clinicopathological characteristics were determined for subjects with cytokeratin-positive cells in the bone marrow. Of these 45 patients, 9 (20.0%) had cytokeratin-positive cells in the bone marrow at the time of primary surgery. These positive findings were not related to tumor advance-related factors of lymph node metastasis and distinct lymphatic and vascular invasion. Microvessel density in the primary tumor exceeded 2-fold in cytokeratin-positive cells, compared with findings in negative cells (P < 0.05). Tumor cells in bone marrow are indicative of the general disseminative metastasis in patients with early gastric cancer, and the metastatic potential was closely related to angiogenesis in the primary tumor.

INTRODUCTION
Early gastric cancer is defined as a lesion in which the depth of invasion is limited to the mucosa or mucosa and submucosa, regardless of whether a regional lymph node metastasis is evident on histological examination, and the postoperative prognosis is usually favorable (1, 2). The diagnosis of early gastric cancer is much more frequent in Japan because sophisticated diagnostic techniques are now available (3).

However, even after “curative” resection of early gastric cancer, there are recurrences in a subset of patients (4–8). Hematogeneous metastasis is the predominant route in such cases. It is difficult to control tumor growth in cases of a distant metastasis in these patients, and knowledge of the stage of subclinical tumor cell dissemination is needed to design adjuvant treatment. Various parameters, including DNA ploidy and oncogenes, aid in predicting the recurrence and the prognosis of early gastric cancer (9–12). A feature common to all of these prognostic factors is that, from the excised tumor tissues, one attempts to extrapolate to the malignant potential of occult cells that may possibly be present. Diagnostic techniques presently available are not sufficiently sensitive to detect unicellular or oligocellular micrometastasis.

Because cytokeratin proteins are essential constituents of the cytoskeleton of both normal and malignant epithelial cells, they can serve as reliable markers for the epithelial origin of cells (13). The use of a monoclonal antibody against the cytokeratin component expressed by all tumor cells derived from simple epithelium facilitates identification of one of 105 epithelial tumor cells in bone marrow. Evidence of micrometastasis in the bone marrow means an early relapse and a poorer clinical outcome for patients with gastric, colorectal, mammary, and lung cancers (14–19). Schlimok et al. (20) found no positive cells in a large series of bone marrow aspirates from 102 patients with no evidence of malignant epithelial disease. We reported the presence of micrometastasis in bone marrow for 32.6% patients with gastric cancer (21). However, the presence of micrometastasis was not focused on patients with early gastric cancer.

Angiogenesis is closely involved in tumor progression and metastasis (22, 23). Weidner et al. (24) reported that microvessel density evaluated by immunostaining for endothelial cells was an independent prognostic indicator in breast cancer. The level of microvessel density particularly correlated with hematogeneous metastasis of gastric and colorectal cancers (25, 26). CD-31 is a platelet/endothelial cell adhesion molecule and is a more sensitive marker for endothelial cells than is factor VIII antigen (27, 28). We examined micrometastasis in bone marrow from patients with early gastric cancer, using an anti-cytokeratin monoclonal antibody, and we determined the microvessel density in the primary tumor, using an anti-CD31 monoclonal antibody. Bone marrow aspirates were taken immediately prior to the initial surgery done from 1994 to 1997.

PATIENTS AND METHODS
Patients. This study included 45 unselected Japanese patients with primary early gastric cancer, all of whom underwent curative gastric resection in the Department of Surgery II, Kyushu University, from 1994 to 1997. The depth of invasion
was mucosa in 27 patients and mucosa and submucosa in 18 subjects. Standardized procedure was that a gastric resection was done, after determining the resection line 3 cm apart from the macroscopic edge of the localized tumor, and 6 cm for the infiltrative tumor (29, 30). Prophylactic lymph node dissection of more than D2 resection was carried out (31). Complete excision of invaded organs was done, irrespective of the number of sites on the organs, when there was no evidence of peritoneal dissemination, liver metastasis, and widespread nodal involvement (32). All patients were examined clinically and pathologically with respect to the factors given in Table 1. Pathological diagnosis and classification of the resected gastric cancer tissues were made according to the General Rules for the Gastric Cancer Study in Surgery and Pathology in Japan (33, 34). Informed consent to participate in this study was obtained from all patients prior to their surgery.

**Bone Marrow Specimens.** Details are given in our study published previously (21). Preoperatively, 1–2 ml of bone marrow aspirates from the sternum were taken in syringes containing 100 units of heparin/ml marrow, and bone marrow cells were prepared. After density centrifugation through Ficoll-Hypaque (400 × g for 30 min), mononuclear cells were collected from the interphase. The cells were suspended with 0.5 ml RPMI 1640 containing 10% FCS, yielding a concentration of 2 × 10⁸/ml; the cells were then smeared on glass slides and fixed with acetone (30 min at 4°C). For immunostaining, the monoclonal antibody CK2 (IgG1; Boehringer Mannheim, Mannheim, Germany) or CAM 5.2 (IgG₂μ; Becton Dickinson, CA) was used at a concentration of 0.2 μg/ml (17). This antibody recognizes intracellular cytokeratin component no. 18, an intermediate filament representing the intracellular network of the cytoskeleton that is expressed in simple epithelia and nowhere else. The antibody reaction was developed using the labeled avidin-biotin technique (35), and biotin-labeled antibody and alkaline-phosphatase-labeled avidin were used sequentially. Naphthol-AS-BI-phosphate was used as a substrate of alkaline phosphatase, and the released naphthol-AS-BI was coupled with hexazonitized new fuchsin. Endogenous phosphatase was inhibited by preincubation with levamisole. Cells containing cytokeratin were stained bright red. Two observers (Y. M. and Y. K.) examined the positivity of micrometastasis of bone marrow independently.

**Immunohistochemistry of CD31.** Tumors were collected and fixed in 10% formalin. Sections of 5-μm thickness from paraffin-embedded blocks were deparaffinized in xylene and rehydrated in a graded series of ethanol. After quenching the endogenous peroxidase activity in methanol containing 0.3% (v/v) hydrogen peroxidase for 30 min, sections were pretreated with 12.5 mg of protease type XXIV (Sigma Chemical Co., St. Louis, MO)/100 ml PBS (pH 7.4) for 15 min at 37°C. Nonspecific binding was blocked by treatment with 10% (v/v) normal goat serum for 15 min at room temperature. After a thorough washing in PBS, biotinylated goat anti-mouse immunoglobulin G (Vector Laboratories, Burlingame, CA) diluted 1:200 was applied, followed by incubation for 30 min at room temperature. After washing in PBS, streptavidin-conjugated streptavidin (DAKO LSAB kit; DAKO) was applied, and the preparations were incubated for 30 min. Peroxidase labeling was developed with diaminobenzidine and hydrogen peroxidase, and nuclear counterstaining was performed with Mayer’s hematoxylin solution (Fig. 1). Specificity of binding for all antibodies was examined by applying nonimmune sera instead of specific antibodies.

**Microvessel Counting.** Evaluation of microvessel density (as a continuous variable) was determined, using the modified technique of Weidner et al. (24). The entire tumor section was systematically scanned at ×100 to search for areas of the most intense neovascularization; these were identified as having the highest density of red-brown staining, CD31-positive cells or cell clusters. These neovascular “hotspots” were included into the counts only if they were adjacent to tumor tissue. Whenever a highly vascularized area was evident at ×100, individual microvessels were counted on a single ×200 field (1 mm²) in this area. Any red-brown-staining endothelial cell or endothelial cell clusters, clearly separated from adjacent microvessels, were regarded as a single, countable microvessel. Neither vessel lumens nor RBCs were used to define a microvessel. Immuno-positive macrophages and plasma cells were excluded on

### Table 1: Clinicopathological characteristics of patients with early gastric cancer with and without cytokeratin-positive cells in the bone marrow

<table>
<thead>
<tr>
<th>Gender</th>
<th>Cytokeratin-negative cases (n = 36)</th>
<th>Cytokeratin-positive cases (n = 9)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>25</td>
<td>6</td>
<td>NS</td>
</tr>
<tr>
<td>Female</td>
<td>11</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Age (yr)</td>
<td>60.9 ± 1.8b</td>
<td>56.4 ± 6.8b</td>
<td>NS</td>
</tr>
<tr>
<td>Tumor maximal diameter (cm)</td>
<td>2.81 ± 2.14b</td>
<td>2.52 ± 2.26b</td>
<td>NS</td>
</tr>
<tr>
<td>Location of tumor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper</td>
<td>9</td>
<td>2</td>
<td>NS</td>
</tr>
<tr>
<td>Middle</td>
<td>17</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Lower</td>
<td>10</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Differentiated</td>
<td>22</td>
<td>4</td>
<td>NS</td>
</tr>
<tr>
<td>Undifferentiated</td>
<td></td>
<td>14</td>
<td>5</td>
</tr>
<tr>
<td>Depth of invasion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucosa</td>
<td>21</td>
<td>6</td>
<td>NS</td>
</tr>
<tr>
<td>Submucosa</td>
<td>15</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>30</td>
<td>6</td>
<td>NS</td>
</tr>
<tr>
<td>Positive</td>
<td>6</td>
<td>3</td>
<td></td>
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<tr>
<td>Lymphatic invasion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>31</td>
<td>8</td>
<td>NS</td>
</tr>
<tr>
<td>Positive</td>
<td>5</td>
<td>1</td>
<td></td>
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<tr>
<td>Vascular invasion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>31</td>
<td>8</td>
<td>NS</td>
</tr>
<tr>
<td>Positive</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Gastric resection</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Partial</td>
<td>25</td>
<td>7</td>
<td>NS</td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Lymph node dissection</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>D1</td>
<td>2</td>
<td>1</td>
<td>NS</td>
</tr>
<tr>
<td>D2 and D3</td>
<td>34</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

a NS, not significant.
b Mean ± SD.
c D1, complete removal of group 1 lymph node alone; D2, complete removal of groups 1 and 2 lymph nodes; and D3, complete removal of groups 1, 2, and 3 lymph nodes.
morphological grounds. Results were expressed as the mean of values in five fields.

**Postoperative Chemotherapy.** All of the patients with cytokeratin-positive cells were treated with postoperative adjuvant chemotherapy. An i.v. injection of 10 mg of mitomycin C (Kyowa Hakko Co., Japan) was given on the day of operation, and fluorinated pyrimidine UFT (Taiho Pharmaceutical Co., Japan; Ref. 38) p.o. at a daily dose of 400 mg was started 2 weeks after the operation and was continued for 1 year.

**Statistical Analysis.** The BMDP Statistical Package program (BMDP, Los Angeles, CA) for the IBM 3090 mainframe computer was used for all analyses (39). The BMDP P4F and P3S programs were used for the \( \chi^2 \) test and the Mann-Whitney test to compare data on patients with and without cytokeratin-positive cells in their bone marrow. The level of significance was \( P < 0.05 \).

**RESULTS**

Cytokeratin-positive cells were present in 9 of 45 (20.0%) patients with early gastric cancer, the depth of penetration being the mucosa or mucosa and submucosa. Alkaline phosphatase-stained cells in cytocentrifuge preparations varied from a single cell to a cluster of 10 cells, as determined histologically. Cytokeratin-positive cells were confirmed to be cancer cells, determined using Papanicolaou staining. Seeding of cancer cells had already occurred in these cases, even though extensive lymph node dissection had been done and a surgically “curative” operation was carried out.

In a patient with mucosal gastric cancer, the tumor measured only 0.9 x 0.6 cm, was a macroscopically depressed type (IIc), localized in the angle of the stomach, and was limited to the mucosa (Fig. 2, A and B). In this patient, a small cluster of cytokeratin-positive cells was present in bone marrow aspirates (Fig. 2C).

**Micrometastasis in the Bone Marrow and Clinicopathological Factors.** Positive findings of micrometastasis in the bone marrow of patients with early gastric cancer did not depend on gender, age, tumor size, tissue differentiation or location of tumor (Table 1). The depth of penetration was the mucosa and mucosa and submucosa, and the tumor size was smaller. The rates of lymph node metastasis and distinct lymphatic and vascular involvements were low, and these events were not related to the presence of micrometastasis. There were evidently no cytokeratin-positive micrometastatic cells in the lymph nodes.

**Micrometastasis in the Bone Marrow and Microvessel Density.** Microvessel counts determined by anti-CD31 antibody in primary gastric cancer tissues were 13.5 ± 6.0 for the cytokeratin-negative patients and 27.9 ± 8.3 for the cytokeratin-positive ones, with a statistical significance of \( P < 0.05 \) (Table 2). Thus, the presence of micrometastasis in the bone marrow showed a close relation to tumor angiogenesis.

**DISCUSSION**

Even after curative resection, there can be recurrences of the cancer in patients with early gastric malignancy (4–8). The most frequent mode of recurrence is by a hematogenous spread, and tumor cells have already disseminated to distant organs at the time of surgery. Availability of a highly sensitive method would aid in predicting metastatic potential and clinical outcome, and more effective treatments could be designed. Clinicopathological factors of age of the patient, tissue differentiation, growth pattern of pen A type, and nodal involvement were reported to be prognostic for the occurrence of recurrences.
A

B

C

Fig. 2 Resected tissue specimen and microphotographs from a patient with mucosal gastric cancer. Macroscopic size of the tumor was 0.6 × 0.9 cm and the depressed type (A). Cancer cells were limited in the depth of the gastric mucosa (B; X 18). High-power photomicrograph of bone marrow specimen reveals a small cluster of malignant cells (C; X1000).

in early gastric cancer (1, 7, 40). Gastric cancer markers may provide prognostic information independent of and complementary to conventional parameters, including growth potential, oncogenes, tumor-suppressor genes, and DNA flow cytometry, as well as other growth factors (9–12, 41). The common characteristic of these prognostic factors is that they correlate a property of the primary tumor with the subsequent outcome.

The method we have described here relates to aspects of the actual behavior of the tumor, microscopic dissemination of cancer cells in the bone marrow. There are reports of micrometastasis in the bone marrow of patients with gastric, colorectal, breast, prostate, or lung cancers (13–19, 42). Evidence of micrometastasis means an early relapse, and the clinical outcome for these patients can be predicted. We also found cytokeratin-positive cells in bone marrow of our patients with gastric cancer, and the micrometastasis in the bone marrow did not correlate with p53 overexpression and proliferating activity of the tumor (21).

Micrometastasis was noted in 20.0% of our patients with early gastric cancer; thus, seeding of cancer cells can occur even in the early stages of the cancer. In these cases, small tumors are just an early manifestation of systemic disease, and metastases has already occurred. However, the cancer cells were single to at most 10, and a few cells were present in bone marrow; however, vascular involvement in primary tumor or lymph node metastasis is difficult to identify.

Tanigawa et al. (25, 26) reported that tumors that developed hematogeneous metastasis after surgery had significantly higher microvessel density than did tumors with related peritoneal metastasis and nonmetastatic tumors. Vascularization is usually required for tumor cells to enter the blood circulation (23). Newly formed tumor vessels are devoid of or lacking in smooth muscle, are tortuous and sinusoidal, have increased vascular length and diameter, have incomplete endothelial cell lining and basement membrane, and are prone to spontaneous hemorrhage and/or thrombosis, thus enabling tumor cells to enter circulating systems (24, 43). In such cases, cancer cells released from the primary site would be transported to the bone marrow. Because the presence of micrometastatic cells in the bone marrow was closely related to angiogenesis in the primary tumor, detection of micrometastasis in the bone marrow does have clinical significance when attempting to evaluate the hematogeneous metastatic potential of gastric cancer.

A higher sensitivity test to detect micrometastasis in the bone marrow can be achieved by making use of the reverse transcription-PCR (44–46). However, this method needs further standardization, PCR primers specific for each RNA tool have to be designed, the optimal number of PCR cycles has to be defined, as do best cutoff levels, and so on (18, 47).

Overt bone or skeleton metastases are rare in patients with gastric cancer; however, bone marrow is more often involved than expected based on clinical findings (17). The apparent discrepancy between clinically rare bone metastases and the marrow micrometastases frequently detected by immunocytochemistry can be explained by a reduced proliferative behavior of the cells and often invoked state of dormancy (20, 42). The capacity of tumor cells to proliferate in the bone marrow and to manifest metastasis depends on the microenvironment. Jauch et al. (18) reported that positive bone marrow aspirations are a surrogate marker of general tumor-cell dissemination or mini-

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Microvessel density of early gastric cancer with and without cytokeratin-positive cells in the bone marrow</th>
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</thead>
<tbody>
<tr>
<td>Factor</td>
<td>Cytokeratin-negative cases (n = 36)</td>
</tr>
<tr>
<td>Microvessel density</td>
<td>13.5 ± 6.0*</td>
</tr>
</tbody>
</table>

* Mean ± SD.
mal residual disease, rather than the start of metastatic growth in the skeletal system. The survival time was shorter in the cyto-
keratin-positive group than in negative group in cases of gastric cancer (16–18). Therefore, these patients may be at a higher risk for complications arising from peritoneal dissemination or liver metastasis with no manifest clinical metastasis in the bone marrow (2, 48, 49).

Because cytokeratin-positive cells were present in the bone marrow of our patients with early gastric cancer, these cells can serve as valid indicators of the metastatic activity of these cancers. Patients presenting with disseminated cytokeratin-positive cells at the time of primary surgery can be followed to detect any distant metastasis. Therefore, such patients may be good candidates for postoperative adjuvant trials, even when a curative resection is done for patients with early gastric cancer.

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REFERENCES

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