Malignant Potential and Cytogenetic Characteristics of Occult Disseminated Tumor Cells in Esophageal Cancer

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Abstract

Although micrometastatic cancer cells in lymph nodes can be detected by monoclonal antibodies against epithelial or tumor-associated antigens, it remains unclear whether these cells are precursors of overt metastases or shedded tumor cells with a limited life span. Here we used esophageal cancer as a model to evaluate the prognostic significance and biological characteristics of such micrometastases. In lymph nodes classified as tumor free by conventional histopathological staging, tumor cells were identified with monoclonal antibody Ber-EP4 in 89 of 126 patients (71%) with completely resected (R0) esophageal carcinomas. Multivariate survival analysis underlined the strong and independent prognostic significance of Ber-EP4-positive cells in “node-negative” (pN0) patients. To assess the biology of Ber-EP4-positive cells, we established tumor cell lines from an immunohistochemically positive lymph node and the autologous primary tumor. p53 mutational analysis and multiplex-fluorescence in situ hybridization revealed common aberrations shared between both cell lines, whereas an insertion of chromosome 13 material in the short arm of chromosome 1 was only observed in micrometastatic cells. The tumorigenicity and metastatic potential of both cell lines were demonstrated in severe combined immunodeficient mice. In conclusion, our data provide first direct evidence for the malignant potential of micrometastatic cancer cells.

Introduction

Metastatic relapse after the complete resection of an apparently localized primary tumor indicates that at the time of surgery disseminated cancer cells are often undetectable by current tumor staging methods. Therefore, more sensitive immunohistochemical assays capable of detecting occult tumor cells in lymph nodes have been developed, using monoclonal antibodies against epithelial or tumor-associated marker proteins (reviewed in Ref. 1). However, it remains unclear whether these immunostained cells represent viable tumor cells with a metastatic potential or shedded cells with a limited life span or even simply laboratory artifacts. This skepticism is based upon the observation that immunohistochemically identifiable cells lack sometimes the typical morphology of tumor cells (2). On the other hand, the specificity of ultrasensitive nucleic acid-based molecular assays is limited by the lack of any morphological correlate and the low level of ectopic expression of the tumor marker transcript (e.g., carcinoembryonic antigen mRNA) in the surrounding normal leukocytes (3, 4).

In the present investigation, we have analyzed 126 patients with completely resected esophageal cancer, and we provide evidence for a strong and independent prognostic influence of immunohistochemically identifiable tumor cells in lymph nodes. Because the direct analysis of these cells is hampered by their low frequency (10^-4 to 10^-5) we have used the latest culturing methods (5) to establish the first cell line derived from these cells. Subsequent analyses demonstrated, for the first time, that these cells are clonally selected viable tumor cells that are derived from the primary tumor and inherit a tumorigenic and metastatic potential in SCID4 mice.

Patients and Methods

Lymph Node Preparation and Immunohistochemical Detection of Tumor Cells

Lymph nodes, judged as tumor free by the surgeon, were divided into two parts as described previously (6). One part of each lymph node was embedded in paraffin for routine histopathological staining; the other part were snap frozen in liquid nitrogen and stored at −80°C for immunohistochemical analysis. For immunohistochemical detection of tumor cells in lymph nodes, the anti-epithelial cell monoclonal antibody Ber-EP4 (Dako, Hamburg, Germany) was used as described previously (6, 7).

Statistical Analysis

Associations between categorical parameters were assessed via Fisher’s exact test. The Kaplan-Meier method was used to analyze local recurrence-free, distant metastases-free, overall, and relapse-free survival times. For comparison purposes, log-rank tests were performed. Cox’s proportional hazards models were fitted for multivariate analysis (8). Relative risk and 95% confidence limits are presented. Differences between groups are considered significant if the Ps were <0.05 for a two-tailed test.

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Analysis of Genomic Changes, Tumorigenic and Micrometastatic Potential of Immunohistochemically Identifiable Cells

Case Summary. Cells from a primary tumor classified as poorly differentiated adenocarcinoma and from a corresponding histopathologically tumor-free lymph node were sampled for cell culturing for a detailed analysis. The patient’s history can be summarized as follows. The patient underwent radical en bloc esophagectomy with curative intention. In routine histopathological examination, the surgical specimen had tumor-free resection margins (R0), and the tumor was staged as pT3 pN0 M0. In 2 of 38 resected lymph node metastases were found on routine H&E staining. Immunocytochemical analysis of bone marrow aspirates obtained at the time of primary surgery with monoclonal antibody A45B/B3 revealed no evidence for hematogeneous tumor cell dissemination into this organ, which has been implicated as indicator site for blood-borne metastatic cells in esophageal cancer (9). One and a half months after surgery, the patient was readmitted to the hospital because of a large tumor mass in the mediastinum with malignant pleural effusion and a portsite metastasis in the cutis, where a drainage had been placed during primary surgery. No further treatment was intended, and the patient died 2 months after primary surgery.

Generation of Cell Lines from mAb Ber-EP4-positive Cells and the Primary Tumor. Part of one lymph node, derived from the arteria gastrica sinistra and judged as tumor free by H&E staining, and samples of the primary tumor were harvested for culturing. These samples were cut into small fragments, vigorously washed with RPMI 1640 (Life Technologies, Paisley, Scotland) and disaggregated into single-cell suspensions using the Medimachine (Dako, Hamburg, Germany). Cell suspensions were plated in ECM-coated T25 culture flasks (Becton and Lorei, Frankfurt, Germany) and cultured as described previously (5).

DNA Analyses of Cell Lines. Microsatellite analysis and HLA-DRB1* genotyping confirmed that both cell lines were derived from the same patient. Genomic DNA was isolated from cell lines following the protocol of Miller et al. (10). The aqueous DNA solution was frozen at −20°C and thawed for subsequent microsatellite analysis using the GenePrint Fluorescent CTTv STR Multiplex System (Promega Corp., Madison, WI). In addition, genomic DNA was used for HLA-DRB1* genotyping (11).

For p53 sequence analysis of the primary tumor and lymph node micrometastatic cell lines, total RNA was isolated by the method of Chirgwin (12) and purified to poly-A-RNA using the Oligotex mRNA Mini Kit (Qiagen, Hilden, Germany). The reverse transcription of poly-A-RNA was performed using the Superscript II reverse transcriptase (Life Technologies, Inc., Eggenstein, Germany) and random hexameric primers according to the manufacturer’s instructions. The primers 5'-TTTCCA CGA CGG TGA CAG C-3' (nucleotides 154-173) and 5'-CTG TCA TCC AAG TAC TCC ACA CGC G-3' (nucleotides 840-818) as well as 5'-ATG AGC GC TGC TCA GAT AGC-3' (nucleotides 720) and 5'-AAG ACC CAA ACCCA AAA TGG-3' (nucleotides 154-173) were used to amplify p53 cDNA. Depending on the primers used, the entire 1316-bp p53 coding region, or two overlapping fragments which spanned the open reading frame of the p53 cDNA, were obtained. Direct sequencing of PCR products was performed with 100 ng (±0.25 pmol) of purified DNA and 10 pmol of the respective primer. DNA sequencing was performed by the nonradioactive cycle sequencing method using the Taq-DNA polymerase sequencing kit and dye-labeled dideoxynucleotides on a 377 stretch system (Applied Biosystems, Inc., Weiterstadt, Germany) together with a thermocycler (Model 9600; Perkin Elmer, Überlingen, Germany).

FISH. M-FISH (13) was done as described previously (14). In brief, flow-sorted whole chromosome painting probes (kindly provided by Prof. M. Ferguson-Smith, Cambridge, United Kingdom) were amplified by DOP-PCR (15). Probe labeling was performed in a stringent DOP-PCR assay. The probe mix was hybridized for 2 days, and detection, image capturing, and image processing were performed as described previously (14). Results are displayed either as true color, generated simply by overlaying the five source images (Fig. 3A), or classification color (Fig. 3, B, D, and E, third column). FISH with CEPH-YAC 933a5 (kindly provided by Dr. T. Haaf, MPI Berlin, Germany), mapped to 8q24-23-2 (16), was hybridized as described before (17).

SCID Mice Xenograft Assays. For SCID mice xenograft assays, aliquots of 1 × 10^6 to 6.5 × 10^6 viable LN 1590 cells were injected with 200 μl of Matrigel (Becton Dickinson Labware, Bedford, MA) into the flanks of SCID mice. Mice were weighed and monitored for visual and palpable tumors once a week; local tumors were measured in two dimensions. Animals were killed by cervical dislocation and autopsied if the tumor diameter exceeded 15 mm. Bone marrow was rinsed out of both femurs, and the cells were cultured as described above (5). Paraffin-embedded sections of secondary organs were histopathologically analyzed with H&E staining. Care of animals was in accord with guidelines of the Veterinary Department of the University of Hamburg.

Results

Clinical Relevance of Immunohistochemically Identifiable Cancer Cells

A total of 2175 lymph node was sampled from the 126 patients. The clinical data are summarized in Table 1. There was no correlation between the presence of Ber-EP4-positive cells in lymph nodes and the histopathological stage (pT), type, or differentiation grade (G) of the primary tumor (Table 1). As expected, Ber-EP4-positive cells were more frequently detected in patients with overt lymph node metastases (pN1 group) compared with those patients without such metastases (pN0 group; Table 1). In the group of 54 patients classified as pN0, immunohistochemical analyses revealed monoclonal antibody Ber-EP4-positive isolated cells in 30 (56%) patients. For survival analysis, six patients were excluded because they died during their hospital stays (within 60 days after surgery) because of postoperative complications (e.g., respiratory failure, sepsis). From the remaining 48 patients, 20 Ber-EP4-negative-pN0 patients had a median relapse free survival of >55 months compared with 27 months in the 28 Ber-EP4-positive-pN0 patients (P = 0.005; Fig. 1). Most remarkably, only 1 of 20 patients who were found to be free of nodal tumor involvement, as assessed by both histopathological and immunohistochemical analysis, developed recurrence during the observation period compared with 10 of 28 patients with a positive immunohistochemical finding. Similarly, only 1 of 20 patients with Ber-EP4-negative nodes developed recurrence and died, whereas 10 of 28 patients with Ber-EP4-positive nodes relapsed, and 9 of these patients died during the observation period.

In patients with a histopathological pN1 stage, no significant difference was observed in patients in which Ber-EP4-identified additional lymph node involvement compared with patients without additional lymph node involvement for both median relapse-free survival (6 months versus 17 months, P = 0.28) and overall survival (10 months versus 18 months, P = 0.24).

Multivariate Cox regression analysis revealed an independent prognostic influence of immunohistochemically detectable tumor cells in
lymph nodes for both relapse-free survival \((P = 0.01)\) and overall survival \((P = 0.02)\). Furthermore, histopathological lymph node status was also of independent prognostic significance for relapse-free and overall survival, whereas primary tumor stage and grade had no independent influence on patients prognosis (Table 2).

**Analysis of Genomic Changes, Tumorigenic and Micrometastatic Potential of Immunohistochemically Identifiable Cells**

**Generation of Cell Lines from mAb Ber-EP4-positive Cells and the Primary Tumor.** Five nodes of one patient, classified as “tumor free” by routine pathological methods, were screened immunohistochemically for Ber-EP4-positive cells, and this analysis revealed that three of these five nodes were in fact positive (Fig. 2A). A cell line, designated as LN1590, was generated from one of these Ber-EP4-positive nodes that contained 3 Ber-EP4-positive cells per \(10^5\) lymph node cells. Another cell line, designated as PT1590, was generated from the autologous primary tumor using identical culture conditions. Both cell lines have grown by now \(>50\) passages and can therefore be classified as immortal.

**Genomic Characteristics of Immunohistochemically Identifiable Cancer Cells.** The \(p53\) gene was sequenced in both cell lines and the same point mutation \(G \rightarrow A\) at position 738, resulting in a histidine to arginine exchange (His\(^{176} \rightarrow\)Arg\(^{176}\); data not shown).

Both tumor cell lines were in the near-triploid range. Examples of representative M-FISH karyotypes are shown in Fig. 3A. Both cell lines shared some common structural rearrangements including del(5)(q1?3q2?2), der(8), der(9)del(9)(::p21~22~q21~22), der(17), der(22)t(19;22), and loss of the Y chromosome (Fig. 3, A and B). An 8q24.2-3 band-specific YAC yielded signals that were detected on both ends of the der(8) (Fig. 3C). Together with the \(4\',6\'-diamidino-2-phenylindole\) bands, the structure of the der(8) was determined as der(8)(qter~q1?::p2?~qter). Further aberrations were observed exclusively either in the primary tumor cells or in the micrometastatic cells. In the primary tumor cells, five structural changes, der(1)(t(1;20), der(3)(t(3;17), der(8)(t(5;8), der(10)(t(1;10), and der(13)(5;13), could be observed in \(\sim 50\%\) of metaphase spreads, which were not seen in the micrometastatic cells (Fig. 3D). Only the micrometastatic cells showed an insertion of chromosome 13 material in the short arm of chromosome 1, resulting in a der(1)ins(1;13)(p22; q?) (Fig. 3E). In addition, some structural aberrations were observed in both cell lines that occurred in a single metaphase only (data not shown).

**Tumorigenic and Micrometastatic Potential of Immunohistochemically Identifiable Cancer Cells in SCID Mice.** To assess the *in vivo* tumorigenic potential of LN1590 cells, between \(1 \times 10^5\) and \(6.5 \times 10^5\) cells were transplanted s.c. into the lateral flanks of nude immunodeficient SCID mice. After 2–29 weeks of observation (mean, 13.8 weeks), 12 of 13 mice developed local tumors at the site of injection (Fig. 2B). In two animals, the local tumors invaded through the peritoneum into the abdominal cavity. Macroscopic metastases into the lung were detected in 5 animals. Furthermore, we were able to re-establish tumor cell lines from different murine tissues, such as mesenterial lymph nodes, bone marrow, and lung, despite the lack of visible signs of metastases. This observation indicates the presence of occult micrometastases in these organs.

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<th>Relapse-free survival</th>
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<td>Relative risk</td>
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<td>Lymph node</td>
<td>Immunohistochemistry (Ber-EP4(^+) vs. Ber-EP4(^-))</td>
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<td></td>
<td>Histopathology (pN(_1) vs. pN(_0))</td>
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\(^{a}\) CI, confidence interval.
Discussion

In this study, we provided both indirect and direct evidence for the malignant potential of immunocytochemically identifiable cells in histopathologically negative lymph nodes. Compelling indirect evidence is provided by the observation that the presence of Ber-EP4-positive cells in histopathologically negative nodes is an independent indicator for a poor prognosis. Direct evidence is provided by a cell line established from an immunohistochemically positive but histopathologically tumor-free lymph node. There are two lines of evidence that this cell line consists in fact of descendants of the autologous primary tumor: (a) the same p53 mutation was observed in both the primary tumor and the cell line but not in normal cells of the patient; and (b) common cytogenetic changes highly specific for this tumor entity were found.

The published data about cytogenetic changes in esophageal cancer are limited, but recent comparative genomic hybridization studies indicated 8q as the most common region of gain and 5q12-21 as the most common region of loss (18). The 8q overrepresentation correlates well with the der(8)(qter→q12):p21→22→q21→22). The 5q loss correlates with the del(5)(q13q22) observed in both cell lines, PT1590 and LN1590. Recently, a gain of 8q23→pter was even associated with lymph node metastasis (19). Other findings, such as the involvement of the short arm of chromosome 22 as seen in the der(22)(ter19:22) or the loss of the Y chromosome, also fit very well with observations published previously by others (18, 20, 21).

It is obvious that this approach has the potential to unravel loci in the genome, which are specifically associated with metastasis. The insertion of 13q material into the short arm of chromosome 1 seen only in the lymph node micrometastases but not in the primary tumor is intriguing because it was reported as frequently involved in structural rearrangements in esophageal cancers (20). This suggests that cells carrying this aberration might have been selected during the process of lymphatic dissemination. The presence of such a micrometastasis-specific anomaly supports the model of clonal metastasis, which implicates that one tumor cell clone disseminates because of a decisive event discriminating it from the other primary tumor cells. After its arrival at the secondary site (e.g., lymph node), this clone needs to undergo further genomic instability to evolve into an overt metastasis.

Our results support the concept that our cell lines are a reliable in vitro system to analyze chromosome-specific aberrations of esophageal cancer and minimal residual disease in particular.

In vitro evidence that occult tumor cells are viable cells with an unlimited proliferative potential is already provided by the generation of such a cell line by itself. To demonstrate whether these cells are tumorigenic in vivo, the cultured cells were transplanted s.c. into immunodeficient SCID mice. Progressive tumor nodules were observed in most of the transplanted animals, proving that the cultured cells were indeed malignant tumor cells.

This is the first report demonstrating that occult tumor cells, detectable with immunohistochemical assays but undetectable by histopathological analyses, are tumorigenic and metastatic in vivo, which implies that these cells might be precursors of subsequent metastatic lesions rather than shedded tumor cells with a limited life span. The controversial discussions about the clinical and biological relevance of micrometastatic tumor cells in lymph nodes of patients with solid epithelial tumors may merely be attributable to assay variability. The Ber-EP4 antibody used in our present study was selected because it is detectable with immunohistochemical assays but undetectable by histopathological analyses, are tumorigenic and metastatic in vivo, and therefore cause false-positive findings. This limited specificity might explain why Glickman et al. (22) failed to demonstrate an independent prognostic impact of cytokeratin-positive cells in lymph nodes of esophageal cancer patients. On the other hand, Luketch et al. (23) used CEA mRNA as marker for esophageal tumor cells and found a significant association to an unfavorable prognosis. Thus,
there is an urgent need for standardization of the current protocols before micrometastatic lymph node staging can be implemented into clinical practice.

In summary, we demonstrated that immunohistochemically identifiable tumor cells present in lymph nodes judged as “tumor free” in routine histopathology are of independent strong predictive value for tumor relapse and overall survival in patients with esophageal cancer. Our observation should have important consequences for tumor staging and therapy. The presence of Ber-EP4-positive cells in lymph nodes should be incorporated in the UICC staging nomenclature by including micrometastases (addition: mi) and isolated tumor cells (addition: i) in the N-category of the Tumor-Node-Metastasis classification. Patients with occult dissemination of viable tumor cells are not cured by surgery alone and may benefit from additional adjuvant therapy. Interestingly, the antibody Ber-EP4, used for tumor cell detection in our study, recognizes the 17-1A antigen (also called epithelial cell adhesion molecule; Ref. 24), which is a promising target for antibody therapy in patients with solid epithelial tumors (1, 25, 26).

References

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