Disseminated Tumor Cells in Pancreatic Cancer Patients Detected by Immunocytology: A New Prognostic Factor\textsuperscript{1}

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ABSTRACT

Using an immunocytological approach, we previously showed that disseminated cancer cells are frequently found in peritoneal cavity and bone marrow samples of gastrointestinal and pancreatic cancer patients. Recently, we demonstrated that the detection of isolated tumor cells could serve as a new prognostic factor in gastric and colorectal cancer. Thus far, no conclusive data concerning the clinical implication of minimal residual disease in pancreatic cancer exist. In this study, we investigated peritoneal lavage and bone marrow samples of 80 pancreatic cancer patients to determine the predictive value of immunocytologically detected disseminated tumor cells. Therefore, immunocytological findings were correlated with the clinical follow-up data (median observation time, 10.7 months; range, 2–61 months), and the findings in peritoneal cavity and bone marrow samples were compared. Fifty-two \% of the patients showed minimal residual disease at least in one compartment (39\% positive lavage and 38\% positive bone marrow samples). The detection rate of isolated tumor cells increased in parallel to the tumor stage. The presence of tumor cells in the peritoneal cavity significantly correlated with the survival time of the patients ($P = 0.0035$). In bone marrow samples, a strong trend was seen ($P = 0.06$). The evaluation of both compartments increased the number of positive patients and resulted in a highly significant correlation: all patients who were positive in at least one compartment died within 18 months, whereas negative patients showed a 5-year survival rate of 30\% ($P < 0.0001$). We recommend immunocytological investigation of peritoneal cavity and bone marrow samples as a new prognostic marker in pancreatic cancer patients.

INTRODUCTION

The prognosis of pancreatic cancer is extremely poor; the overall 5-year survival rate is only 2\%. A chance of cure exists only for a minority of patients with locally limited and surgically resectable tumors (1). However, of patients who are radically treated by a partial pancreatico-duodenectomy and lymphadenectomy (R0 resection), 70–80\% will suffer from an incurable local relapse, distant metastases, or peritoneal carcinoma (2). Although a local relapse might be caused by an incomplete resection, distant metastases and peritoneal carcinoma ultimately depend on a dissemination of malignant cells. Presently, those cells are not detectable with conventional diagnostic tools. However, their elimination is the aim of various adjuvant therapy concepts that are currently under investigation, including chemotherapy (3), immunotherapy (4), and gene therapy (5). For a specific selection of patients who would benefit from those therapies, it would be helpful to detect minimal residual disease.

Immunocytological methods, which are significantly more sensitive than conventional cytology (6), have made it possible to detect disseminated tumor cells in the bone marrow of patients with breast cancer (7), small cell lung cancer (8), neuroblastoma (9), prostatic cancer (10), gastric cancer (11), and colorectal cancer (12). Previously, we showed that the finding of disseminated tumor cells is more likely to occur in the peritoneal cavity because of the high frequency of local relapse or peritoneal carcinosis. Therefore, we also developed an immunocytological approach to detect isolated tumor cells in peritoneal cavity samples (13). Recently, we showed that the finding of tumor cells in the peritoneal cavity can serve as a prognostic marker in gastric and colorectal cancer (16). However, no comprehensive immunocytological studies exist concerning the clinical implication of isolated i.p. and bone marrow tumor cells in pancreatic cancer.

This study was initiated to determine the predictive value of disseminated tumor cells in pancreatic cancer patients. We extended our former studies to a larger collection of patients and investigated peritoneal lavage and, for comparison, bone marrow samples in surgically treated tumor patients and compared the immunocytological findings with the clinical data.

Here, we show that tumor cells were frequently detectable in the peritoneal cavity and in the bone marrow. A finding of isolated tumor cells correlated significantly with the postoperative survival rate of pancreatic cancer patients.

MATERIALS AND METHODS

Patients

All patients were extensively informed and gave written consent for the investigations, including the bone marrow aspi-
Immunocytological Detection of Tumor Cells

We evaluated the data of 80 patients who suffered from an adenocarcinoma of the pancreas and underwent surgery (carcinoma of the papilla vateri and endocrine tumors were excluded). Bone marrow samples were tested in 71 patients (9 patients declined to give their consent), and lavage samples could be obtained in 62 patients (18 patients showed adhesions that were too extensive). Pairs of peritoneal lavage and bone marrow were analyzed in 53 patients.

Patients who died in the hospital due to surgical complications or tumor progress (n = 7) and patients who attended a clinical study for adjuvant therapy (n = 2) were excluded from this study. Another six patients who were initially investigated were lost in the follow-up and, therefore, not included in this study (1 stage II and positive in peritoneal lavage; 2 stage III and immunocytologically negative; and 3 stage IV, 2 of which were lost in the follow-up and, therefore, not included in this study. Another six patients who were initially investigated were lost in the follow-up and, therefore, not included in this study (1 stage II and positive in peritoneal lavage; 2 stage III and immunocytologically negative; and 3 stage IV, 2 of which were immunocytologically positive).

**Control Group.** Fifty-eight patients who underwent surgery but did not suffer from a malignant disease served as a control group: 45 agreed to a bone marrow aspiration and 43 agreed to a peritoneal lavage. The control group included patients suffering from benign liver tumors (n = 10), sigma diverticulitis (n = 8), chronic pancreatitis (n = 7), cholecystolithiasis (n = 5), achalasia (n = 4), other benign diseases (n = 3), and hypersplenism (n = 4). Additionally, ascites from patients with liver cirrhosis (n = 5) and bone marrow samples from patients with benign hematological disease (n = 12) were examined.

**Samples**
Bone marrow (10 ml) was aspirated from the right spina iliaca anterior at the beginning of the operation (Jamshidi needle). Peritoneal lavage was performed before manipulation of the tumor. One liter of isotonic sodium chloride solution was instilled and immediately removed (13).

**Preparation of Samples.** The lavage solution was centrifuged (270 × g for 10 min), and the supernatant was discharged. The cell pellet was resuspended in 20 ml of RPMI 1640. All lavage suspensions and bone marrow aspirates were further processed by Ficoll-Paque (Pharmacia, Uppsala, Sweden) and centrifuged onto microscopic slides (2.5 × 10^5 cells/slide). Cytospins were fixed in acetone and stored at −20°C (13).

**Immunocytochemistry**
Staining of cytospins was performed by the immunoperoxidase method with six different monoclonal antibodies as described previously (13): (a) C1P83, gold 3 epitope of CEA (17); (b) CA19-9 (DAKO, Carpinteria, CA), determinants of Lewis blood group antigens (18); (c) 17-1-A (Centocor, Leiden, the Netherlands), tumor-associated membrane antigen (19); (d) Ra96, tumor-associated mucin antigen (20); (e) C54-0, epithelial membrane antigen (21); because of a cross-reactivity with a subpopulation of lymphopoietic cells in the bone marrow, C54-0 was only evaluated for peritoneal cavity samples; and (f) KL-1 (Immunotech, Hamburg, Germany), cytokeratins (only used in bone marrow samples). The hybridoma cells for the antibodies C1P83, Ra96, and C54-0 were produced by our laboratory, and antibodies were purified according to standard procedures (21). A total of 1.25 × 10^6 peritoneal lavage and bone marrow cells each were tested for every patient. The microscopic evaluation was carried out independently by two investigators. To ensure that the investigators were not influenced by the clinical data, the slides were blinded by numbers, and the investigators were, therefore, not aware of the clinical background of the samples (e.g., tumor stage, control sample, and so on).

**Evaluation of Data**
Samples were evaluated as tumor cell positive if at least one cell reacted with one of the monoclonal antibodies. The detection rate was correlated with the Union International Contre Cancer classification of the tumor stage and the R-classification (22).

Postoperatively, patients were examined either in our outpatient clinic or by their general practitioner. Every 3 months, a clinical examination and blood tests, including tumor markers CEA and CA19-9, were performed, and every 6 months, a sonography or computed tomography scan was performed.

The projected survival rates were determined by Kaplan-Meier calculation, and calculations of significance were determined by the log-rank test.

**RESULTS**

**Control Group.** In 43 of 45 patients, no cell staining was seen in bone marrow samples with the antibodies KL-1, C1P83, RA96, CA19-9, or 17-1-A. In two patients, single cells were stained with the antibodies KL-1, C1P83, and 17-1-A. Additionally, one patient had positive cells for CA19-9. Both patients were strongly suspected to suffer from pancreatic cancer and were, therefore, treated by a partial duodenopancreatectomy (Whipple operation). The histological analysis could not confirm this diagnosis and found a chronic pancreatitis.

The peritoneal lavage samples of patients with no malignant diseases showed, in 40 of 43 cases, no positive reaction of the applied antibodies (C1P83, Ra96, CA19-9, 17-1-A, and C54-0). Two of the positive samples were derived from the same patients mentioned above who suffered from a chronic pancreatitis and showed C54-0- and C1P8-stained i.p. cells, respectively. A third patient with liver cirrhosis and a chronic hepatitis C was positive for the antibody C54-0.

**Pancreatic Cancer.** Overall, minimal residual disease was detected in 42 of 80 (52%) patients; 24 of 62 (39%) had positive peritoneal cavities and 27 of 71 (38%) had positive bone marrow samples. The antibody CA19-9 showed the highest detection rate in bone marrow samples (23%), followed by C1P83 (anti-CEA) and KL-1 (13 and 12%, respectively). In peritoneal cavity samples, C1P83 (31%) and Ra96 (20%) reacted with the highest frequency. As shown previously (13), the combination of all antibodies clearly increased the detection rate in the bone marrow, peritoneal cavity, and combined evaluation (38, 39, and 52%, respectively). On average, we found 5 isolated tumor cells per peritoneal lavage sample (range, 1–95 cells) and 3 isolated tumor cells in the bone marrow (range, 1–5 cells).

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3 The abbreviation used is: CEA, carcinoembryonic antigen.
Fig. 1 shows typical positive results in a peritoneal lavage (Fig. 1a) and a bone marrow sample (Fig. 1b). In peritoneal cavity and bone marrow samples, the detection rate increased in parallel with the tumor stage. Interestingly, one of five (20%) patients with a stage II tumor showed positive cells within the peritoneal cavity, although the tumor had no direct access to the peritoneal cavity; three of eight (38%) presented tumor cells in the bone marrow; and in three of nine (33%), the tumor had spread in both compartments.

A radical partial pancreatico-duodenectomy (Whipple procedure) including extended lymphadenectomy was performed as an R0 resection in 29 patients. In 6 patients, the histological evaluation revealed a R1 resection (microscopically remaining tumor); in 3 patients, a macroscopically incomplete tumor resection was performed (R2 resection); and 42 patients received palliative surgery by abdominal exploration and bypass operation (e.g., gastroenterostomy and biliodigestive anastomosis).

In R0-resected patients, minimal residual disease was detected in 29% of the peritoneal cavity and 21% of the bone marrow samples. In incomplete resected or palliative treated patients, the detection rate was significantly higher and showed tumor cell dissemination in 56% of the peritoneal cavity and in 48% of the...
Table 1  Summary of pancreatic cancer patients (n = 80) who were included in the follow-up study

Shown are the Union International Contre Cancer tumor stage; the number of patients with a “curative” operation (R0 resection), incomplete resection (R1/2 resection), or palliative operation (OP); and the detection rate in the peritoneal cavity (pc), bone marrow (bm), or either compartment (pc and/or bm).

<table>
<thead>
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<th>Stage</th>
<th>R0 resection (n = 29)</th>
<th>R1/2 resection (n = 9)</th>
<th>Palliative OP (n = 42)</th>
<th>Detection rate</th>
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<td>pc only</td>
<td>bm only</td>
<td>pc and bm</td>
<td>pc and/or bm</td>
</tr>
<tr>
<td>I</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0/2 (0%)</td>
</tr>
<tr>
<td>II</td>
<td>5 (17%)</td>
<td>1 (25%)</td>
<td>1 (25%)</td>
<td>1/5 (20%)</td>
</tr>
<tr>
<td>III</td>
<td>13 (44%)</td>
<td>0 (0%)</td>
<td>6 (28%)</td>
<td>3/13 (23%)</td>
</tr>
<tr>
<td>IVa</td>
<td>2 (6%)</td>
<td>4 (14%)</td>
<td>3 (14%)</td>
<td>3/6 (50%)</td>
</tr>
<tr>
<td>IVb</td>
<td>3 (9%)</td>
<td>4 (14%)</td>
<td>33 (71%)</td>
<td>17/36 (47%)</td>
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</tbody>
</table>

Bone marrow probes. Stage IVB patients (n = 36) suffered in four cases, from a peritoneal carcinoma, which was in accordance with immunocytological positive results in two patients.

Table 2  Summary of 53 patients in whom peritoneal lavage and bone marrow were investigated in parallel (“pairs”)

Comparison of tumor stage and number of patients (percentages in parentheses) with positive peritoneal cavity samples only (pc only), positive bone marrow samples only (bm only), patients with immunocytological findings in both compartments (pc and bm), and patients with positive findings in either compartment (pc and/or bm).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Detection rate</th>
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<tr>
<td></td>
<td>pc only bm only pc and bm pc and/or bm</td>
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<tr>
<td>I (n = 2)</td>
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<td>II (n = 4)</td>
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<td>III (n = 10)</td>
<td>1 (10%) 4 (40%) 1 (10%) 6 (60%)</td>
</tr>
<tr>
<td>IVa (n = 6)</td>
<td>2 (33%) 1 (17%) 1 (17%) 4 (66%)</td>
</tr>
<tr>
<td>IVb (n = 31)</td>
<td>9 (29%) 7 (23%) 6 (19%) 22 (71%)</td>
</tr>
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Follow-Up. The postoperative survival rate was determined in 80 patients and correlated with the findings of 71 bone marrow, 62 peritoneal lavage samples, and 53 pairs (peritoneal lavage plus bone marrow). Due to the majority of patients who died within the first year, the median observation time was short (10.7 months; range, 2–61 months), with an overall 5-year survival rate of 14%.

According to the Kaplan-Meier calculation, all patients with positive immunocytological findings in the peritoneal cavity and in the bone marrow died within 15 and 20 months, respectively. In contrast, 29% of the patients with negative findings (n = 38) were supposed to survive at least 5 years.

The log-rank test showed significance for the peritoneal cavity (P = 0.0035) and a statistical trend for bone marrow findings (P = 0.06). The calculation became highly significant (P < 0.0001) when the immunocytological results of all 80 patients were correlated with the survival (minimal residual disease in either compartment; Fig. 2).

Interestingly, the pair analysis of 53 patients showed that the survival rate of patients with tumor cell dissemination in both compartments (n = 9) was as bad as that in patients who suffered from isolated tumor cells in either the bone marrow (n = 13) or the peritoneal cavity (n = 12; Fig. 3). The detection of micrometastatic single cells strongly correlated in any group with a worse survival rate compared to patients who had no signs of dissemination (n = 19; P = 0.0012). However, the data also strongly indicate that the combined evaluation of bone marrow and peritoneal cavity increases the number of positive patients. This is mainly due to a significant group of patients in whom only one compartment could be analyzed (Table 2).

A prognostic value of immunocytologically detected tumor cells was also observed within patients who suffered from the same tumor stage. A significant correlation was found in stage III when minimal residual disease was detected either in the peritoneal cavity or the bone marrow (P = 0.0216). It is remarkable that, in this advanced tumor stage, the 3-year survival rate in negative patients was 20%, whereas all positive patients except one died within 11 months. The evaluation of R0-resected stage III cases (n = 13) showed that three of four patients (75%) with positive findings in the peritoneal cavity or the bone marrow died within 1 year, compared to four of nine negative patients (44%).

In stage IV, similar results were found: all positive patients died within 20 months, but 12% survived more than 3 years (P = 0.09; Fig. 4). This statistical trend became significant when only lavage samples were calculated (P = 0.0273). Due to the low number of cases with early stages I and II, a comparative statistical calculation of survival and immunocytology for these early stages was not possible. However, when patients with stage I and II were combined, one of three patients with positive immunocytology died within 1 year, in contrast to one of nine patients with negative staining, indicating that the detection of isolated tumor cells also serve as an prognostic factor in early cancer stages.

Additionally, we were interested in the prognostic value of each antibody alone. A highly significant correlation with the survival and peritoneal cavity findings was found for C1P83 (P < 0.0001), Prognostically relevant cells were also found with CA19-9 (P = 0.0224), Ra96 (P = 0.04), and 17-1-A (P = 0.0064). Only the antibody C54-0 did not show a significant correlation (P = 0.1242).

In bone marrow samples, Ra96-positive patients had a significant worse prognosis (P = 0.0324). Although CA19-9 showed a statistical trend (P = 0.1115), none of the other...
antibodies could define a significant correlation of positive immunocytology and survival.

DISCUSSION

Immunocytological techniques have made it possible to detect disseminated tumor cells in the bone marrow of various cancer patients. Most studies have been performed with breast cancer patients using a specific antibody for epithelial cells to detect a tumor cell spread in the bone marrow at the time of operation (23). A strong correlation between tumor cell detection and survival could be seen, and hence, these patients, the finding of isolated cancer cells may serve as a new prognostic marker (7). Further studies were published describing a similar approach to search for isolated tumor cells in the bone marrow of lung cancer (8), prostatic cancer (10), and neuroblastoma (9) patients. However, in contrast to the mentioned malignancies, bone metastases are a rare event in pancreatic cancer, the majority of the patients suffer from intraabdominal spread (15) and peritoneal carcinosis (24).

Therefore, we investigated peritoneal cavity samples of pancreatic cancer patients in addition to bone marrow aspirates by an immunocytological approach. Previously, we showed that our approach allows a highly specific tumor cell detection in the bone marrow and in the peritoneal cavity (13). This finding was confirmed in this study. The enlarged control group contained only two positive bone marrow and three positive peritoneal lavage samples. Two of these patients were treated by a Whipple resection due to the strong suspicion of a pancreatic cancer. A third patient suffered from chronic hepatitis C and liver cirrhosis and showed lavage cells that were positive for C54-0. The chance of nonspecific mesothelial cell staining by antibodies directed against tumor-associated antigens has been described (25), but obviously, it is low with the applied antibody panel. Additionally, a further explanation for the detection of disseminated cells in the control group might be that those cells are “disseminated” benign cells. This theory receives some supporting evidence from PCR studies. It was found that normal liver cells were detectable in blood samples of patients who were surgically treated for benign liver disease (26). Using a CK20 nested reverse transcription-PCR, which detects disseminated epithelial cancer cells with a high specificity in the bone marrow of colorectal cancer patients, we (27) found, in rare cases with nonmalignant disease, disseminated epithelial cells in bone marrow and blood samples. Interestingly, one of the “control patients” with a CK20-positive bone marrow sample mentioned in that study is identical to one patient in our study who showed CA19-9-stained cells in the bone marrow. The finding of disseminated cells by two different approaches strongly suggests that no nonspecific cross-reaction with normal bone marrow cells occurred.

However, in our study, the finding of disseminated cells without a proven malignancy was a rare event, and overall, this approach was defined to be highly specific for the detection of isolated tumor cells in pancreatic cancer patients. The specificity of the immunocytological results was supported by the observation that, in most tumor patients, at least two different tumor-associated antigens were stained.

Disseminated tumor cells were i.p. found, even in the 20% of patients with an early tumor stage II in whom a direct tumor access to the peritoneal cavity could be excluded. Because studies in gastric cancer patients suggest those cells most likely reach the peritoneum by pores and lymph vessels and obviously become frequently detectable with high sensitive methods such as immunocytology (13, 28). By finding minimal residual disease in peritoneal lavage and bone marrow samples even in early stages, our study give strong evidence that tumor cell spread is a general and an early feature of pancreatic cancer.

Whether isolated disseminated tumor cells possess the ability to form metastatic disease and are, therefore, of prognostic significance is still controversial. Immunocytological studies concerning the predictive value of isolated gastrointestinal tumor cells exist for gastric, colorectal, and esophageal cancer. All studies suggest isolated tumor cells to be a prognostic factor (16, 29–31). Thus far, only one study investigated the prognostic value of epithelial cells in the bone marrow of pancreatic cancer, but the number of patients in that study was low, and therefore, the follow-up data were not conclusive (32).

Our study is the first comprehensive analysis on pancreatic cancer patients. We clearly showed a prognostic dependence of the survival from minimal residual disease. In accordance to recent
results in gastric and colorectal cancer (16), the predictive value of i.p. cells was superior to findings in the bone marrow. Interestingly, the pair analysis of 53 patients (Fig. 3) strongly indicates that the predictive value of i.p. isolated tumor cells is not improved by an additional finding of cells in the bone marrow. However, due to a significant number of patients (in our study, almost 20%) in whom a lavage could not be performed, the investigation of the bone marrow helped to increase the number of patients with a prognostically significant minimal residual disease. The higher prognostic value of i.p. tumor cells is not fully understood, but it may be that the contact of tumor cells with peritoneal cells support their ability to develop the full metastatic phenotype (e.g., by secreted growth factors). Gastrointestinal epithelial cells are displaced in the bone marrow and may be in the “wrong” environment and, consequently, kept in a dormant state, as Pantel et al. (33) suggested. Further studies will focus on the characterization of the isolated cancer cells to elucidate local factors that may be important in the progress of metastatic disease.

The metastatic potential of isolated tumor cells and, thereby, their prognostic impact became even more evident when patients with the same tumor stage were compared. A Kaplan-Meier calculation was possible for stages III and IV. In both stages, a positive immunocytochemical result in lavage and/or bone marrow probes significantly correlated with the survival. It is remarkable that all positive patients in stage III and in stage IV died within 18 months, but even in stage IV, some negative patients survived at least 3 years. Furthermore, in stage III patients who received a curative R0 resection, three of four (75%) died within 1 year when minimal residual disease was detected. In contrast, only four of nine (44%) negative patients died within this period.

Due to the low number of positive patients, in stage I and II, a Kaplan-Meier calculation was not performed, but the trend in these early tumor stages was similar to stage III and IV: one of three patients with positive findings died within 1 year. In contrast, one of nine patients in the negative group died during this period from tumor relapse (5-year survival time, 70%; data not shown).

By using a significantly higher sensitive technique, our study gives strong support to a recent cytological study that showed a worse prognosis of pancreatic cancer patients suffering from an early i.p. dissemination (34) and can have direct clinical implications: patients who are immunocytochemically tested negative might benefit from a more aggressive surgical approach, which is currently not recommended in most stage III and IV patients. On the other hand, immunocytochemically positive tested patients, especially patients with tumor cells, have an extremely poor prognosis. A radical surgical approach with a risk of high morbidity seems to be legitimate only if, postoperatively, an effective (and systemic) adjuvant therapy can be offered in the future.

In summary, using an immunocytochemical approach, we demonstrated that minimal residual disease becomes frequently detectable in the peritoneal cavity and the bone marrow of pancreatic cancer patients. The occurrence of isolated tumor cells correlates with a poor prognosis and, thereby, serves as a new prognostic marker. This technique might be helpful in guiding surgical therapy and new adjuvant treatment concepts.

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